

**THE EFFECT OF ENZYME- OR RADIATION-INDUCED
DOUBLE STRAND BREAKS ON THE SURVIVAL OF
PLASMID AND VIRAL DNA**

THESIS

submitted in fulfillment
of the requirements for the
Doctorate Degree (Ph. D.)
in Biology
from Faculty of Biology
Ruhr-Universität Bochum

By
Malak Zahran
(Cairo Egypt)

1989



THE EFFECT OF EXTENSIVE EXERCISE-INDUCED
DOUBLED STRAIN STRESS ON THE REMOVAL OF
HARVEST AND WIND DNA

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THE EFFECT OF RADIATION ON POLYMERIZATION
DOUBLE STRUNG EFFECTS ON THE SURVIVAL OF
PLANTS AND THEIR DNA

Abstract of the
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the Biological Effects of Ionizing
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"Gedruckt mit Unterstützung des Deutschen
Akademischen Austauschdienstes"

TO MY HUSBAND

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Malak Zahran

ABBREVIATIONS AND NOMENCLATURE

ATP	Adenosine-5'-triphosphate
Amp	Ampicillin
Amp ^r	Ampicillin resistant
Amp ^s	Ampicillin sensitive
APS	Ammonium peroxydisulphate
bp	base pair
bd	base damage
ccc DNA	covalently closed circular DNA
CsCl	Cesium chloride
Ci	curi
dATP	2'-deoxyribosyladenine 5'-triphosphate
dCTP	2'-deoxyribosylcytosine 5'-triphosphate
dGTP	2'-deoxyribosylguanine 5'-triphosphate
DNA	Deoxyribonucleic acid
dTTP	2'-deoxyribosylthymine 5'-triphosphate
ddNTP	dideoxyribosylnucleotide 5'-triphosphate
ddATP	dideoxyribosyladenine 5'-triphosphate
ddCTP	dideoxyribosylcytosine 5'-triphosphate
ddGTP	dideoxyribosylguanine 5'-triphosphate
ddTTP	dideoxyribosylthymine 5'-triphosphate
ds	double stranded
dsb	double-strand break(s)
E.coli	Escherichia coli
EDTA	Ethelene diamine tetraacetic acid
EtBr	Ethidium Bromide
Gy	Gray
HPLC	High performance liquid chromatography
KB	Kilobases
Lin	Linear
min	minute(s)
Mw	Molecular weight
pBR322/BamHI	pBR322 digested with BamHI (linear)
pBR322/PvuII	pBR322 digested with PvuII (linear)
pBR322/PstI	pBR322 digested with PstI (linear)



pBR322/SalI	pBR322 digested with SalI (linear)
pBR322/NruI	pBR322 digested with NruI (linear) and etc.
rec-mutation:	mutant with defect in recombination gene.
RNA	Ribonucleic acid
RNase	Ribonuclease
Sc	Supercoiled
ss	single stranded
ssb	single-strand break(s)
SDS	Sodium dodecyl sulphate
TBE	Tris-Borate-EDTA
Tet	Tetracycline
Tet ^r	Tetracycline resistant
Tet ^s	Tetracycline sensitive
TE	Tris-EDTA
TEMED	N,N,N',N'Tetramethyl-ethylene-diamine
TM	Tris-Magnesium
Tris	Tris hydroxymethyl aminoethane
UV	Ultraviolet

THE EFFECT OF ENZYME- OR RADIATION-INDUCED DOUBLE STRAND BREAKS ON THE SURVIVAL OF PLASMID AND VIRAL DNA

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CHAPTER 1

INTRODUCTION

The integrity of DNA is essential for the well-being of a cell. Damage to this molecule, whether caused by reaction with chemical mutagens, by irradiation with ultraviolet (UV) light, or by ionizing radiation, has dire consequences. Exposure of DNA to high-energy radiation such as gamma-rays can undergo considerable changes due to chemical reactions induced by the ionizations and excitations of the molecules. Because of this property ionizing radiation is applied in many fields such as production of new materials, food preservation and cancer therapy, but is also found to be harmful to human health forcing to rigid radiation protection measures. Therefore, it is not surprising that in the field of radiation research much attention is paid to the biological consequences of radiation by studying its effects in a large variety of objects from organisms to biomolecules. Many of such studies are dedicated to the DNA molecule, carrier of all genetic informations for protein production and for cell regulation. Irradiation of this vital molecule produces damage which can cause mutations, reproductive cell death and the disappearance of some or all cell activities. When a living cell or DNA molecule is subjected to ionizing radiation, ions, free radicals and excited molecules will be created. Therefore, two kinds of formation of DNA damage are often distinguished, the so called "direct" and the "indirect" effect of irradiation. The direct effect results from absorption of the radiation energy by the molecule in which the lesion occurs i.e. due to ionization or electronic excitation of DNA. The indirect effect is caused by absorption of the energy by surrounding molecules (such as H_2O) which are altered and subsequently react with the molecule in which the lesion is observed. In other word, the indirect effect is caused by reactive species, in most cases free radicals, which are produced in the vicinity of the DNA. Absorption of ionizing radiation in aqueous solutions gives rise to OH and H radicals and hydrated electrons. All of these

radicals are very reactive with DNA causing electron addition and H and OH addition, as well as H abstractions which can alter the molecule in variety of ways. OH radicals are responsible for most of the indirect contribution to the damage in DNA and in turn its biological inactivation in the cell. In DNA that has been exposed to ionizing radiation, four types of damage can be distinguished: single-strand breaks, double-strand breaks, cross-links and nucleotide damage. The latter comprises all damage in the heterocyclic bases and sugar moiety which is not associated with chain breakage or cross-linking.

Double-strand breaks are produced in the dsDNA molecule by the action of specific restriction enzymes. These enzymes are endonucleases and strain-specific which enable bacteria to recognize and rapidly destroy foreign DNA and which cause double-stranded scissions at a limited number of sites on the DNA. The restriction endonucleases have played an essential role in the current position of DNA manipulation in the biological sciences and the development of the field of recombinant DNA technology. These enzymes catalyse the hydrolysis of ds DNA with a high level of specificity to generate a well-defined series of polynucleotides called restriction fragment. The enzymes recognize specific sequences within DNA molecules and hydrolyze the phosphodiester bonds within or near their particular recognition sequences. These sequences typically are four or six nucleotides in length and show dyad symmetry. Restriction enzymes may generate fragments with blunt or sticky ends. Three kinds of restriction endonucleases, designated type I, II and III, are currently known. Enzymes of the most important type II bind at specific recognition sequences and also digest the DNA in an absolutely site-specific reaction to yield well-defined DNA-fragments (or linear DNA by a unique restriction). The only cofactor required by enzymes of this type is Mg^{2+} . The advantage of restriction endonucleases performing is that the enzymes act on known unique position on the DNA molecule and give only one dsb resulting in linear DNA (homogenous damage). For the study of two types of dsDNA (viral and plasmid), we used differnt

restriction endonucleases which give a unique dsb covering mostly the DNA molecule.

The contribution of single- and double strand breaks to the inactivation of ϕ X174 bacteriophage DNA has been investigated extensively (Blok and Loman, 1973; Schans *et al.*, 1973 and Lafleur and Loman, 1986). This DNA is a suitable subject for the study of the effects of damaging factors in connection with biological inactivation. The advantage is that the DNA can be used in single stranded or double stranded forms and possesses biological activity which can be measured simply and quantitatively. The DNA molecule contains 5386 nucleotides in length (Sanger and Coulson, 1975, see Appendix)). In the present study the two forms of ϕ X174 DNA were used in order to study the effect of γ -rays while the dsDNA form was used for studying the effect of restriction endonucleases.

Because of their small size, stable autonomous maintenance and dispensable nature, bacterial plasmids offer an attractive model system for studying the mechanism by which the DNA damage and repair is proceeded. Plasmids are widely used as vectors for cloning various types of DNA in bacteria. Among these is pBR322 plasmid which composed of three major parts, i.e., a region of Tn3 and a region of pSC101 which specify ampicillin and tetracycline resistance, respectively, and a region of pMB1 which carries the origin of DNA replication (*ori*). The sequence of the plasmid pBR322 was known (Sutcliffe, 1979 and Peden, 1983) and the total length is 4363 bp (see Appendix). The presence of drug-resistance markers in the plasmid is helpful in measuring the effect of damages on the DNA molecule as well as in the selection of the deletants (mutants) produced. The technique of transformation of *E. coli* cells with biologically active plasmid pBR322 or viral DNA was used in the present study. The advantage of this technique is that the DNA can be damaged (by irradiation or restriction endonucleases) under controlled conditions in aqueous solution and the biological activity can be tested afterwards.

DNA is the only cellular molecule that can be repaired. The uniqueness of such repair reflects the extraordinary importance of DNA to cellular life. If the genetic message is defective, everything that follows will also be defective, and a mutation in the wrong place can be fatal. A variety of repair mechanisms are known. Except for photoreactivation of the thymine dimers, all known modes of DNA repair operate by excision repair. In general excision repair requires four steps: *incision*, that is, formation of a nick in the dsDNA; *excision* by cleavage of a second phosphodiester bond to give a gap in dsDNA; *synthesis* of new DNA to replace the deleted region; and *insertion* of the new DNA and *sealing* of the nick. The base and nucleotide damage can be repaired by this mechanism. Recombination is the major mechanism for Lin DNA repairing. Genetic analysis revealed that the repair of dsb of the *E. coli* genome is due to recombinational event. The linearized DNA (by enzymes or irradiation) which is introduced into *E. coli* cell by transformation can be repaired by the enzymes of the host (recombination is usually involved, Thompson and Achtman, 1979 and Conley *et al.*, 1986a). Recombination can be carried out via *RecBC*, *RecF*, or *RecE* pathways which depends on special gene products (Symington *et al.* 1985). The ability of *E. coli* to recircularize linear DNA molecules *in vivo* by recombination process explains the generation of many of the aberrant recombinant DNA molecules that are detected in gene cloning experiments (Conley *et al.*, 1986b).

Despite the many years of research dedicated to restriction endonucleases and radiation effects of biological interest, our knowledge about the fundamental processes leading to such effects and their repairing is still limited. Furthermore, the biological relevance of the different damaged products which have been produced by the action of restriction enzymes and γ -rays on DNA is still unknown. The aim of the study described in this thesis to learn more about the mechanisms which underlie the induction of biologically relevant radiation and

restriction damage in plasmid and viral DNA. In the study we report data on the surviving of ϕ X174 DNA containing dsb produced either by restriction endonucleases or by γ - irradiation. The type of damage in connection with repairing efficiency of dsDNA in comparison with the ssDNA form was discussed in Chapter 2. In the following sections, the repair of pBR322 DNA containing different types of dsb produced by different restriction endonucleases was reported. The role of the restricted gene and termini on the re-cyclization of linear plasmid was reported (Chapter 4). It was interested to follow the miniclones (mutants) which were found to appear with the normal clones after transformation of the linear plasmid molecules. The properties of these miniclones and their contents of pBR322 plasmid DNA was studied in details in Chapter 5. To know a definite information about the structure of plasmid in the other mutants, which appear as normal-size clones, the sequencing technique was performed for plasmid DNA of three different tetracycline resistant transformants. The role of restricted ends and the matching homology sequences in the plasmid molecule in connection with the intramolecular recombination processes and the formation of deletions was givin in Chapter 6. The study gives more informations about the surviving of linear plasmid and viral DNA by repairing the dsb of these molecules in *E. coli* cells.

CHAPTER 2

MATERIALS AND METHODS

I-MATERIALS APPENDEX

Buffers and Solutions:

40% Acrylamide	380 g acrylamide, 20 g bisacrylamide/1L.
Chase	0.25 mM dNTP's (4 deoxyribonucleotides).
Dye solution	20 g sucrose, 10 ml 10xTBE, 0.1 ml bromophenolblue/100 ml.
FM stop solution	100 ml formamide, 0.1 g xylene, 0.1 g bromophenolblue, 5 ml (0.2 M) EDTA.
Gel-fix	10% methanol, 10% acetic acid/1L solutions.
Lysozyme	2 mg enzyme/ml Tris buffer (0.25 M) pH 8.1.

ddNTP solutions:

	(sequencing solution)
ddATP	500 μ l 0.5 mM dTTP + 500 μ l 0.5 mM dCTP + 500 μ l 0.5 mM dGTP + 3 μ l 10 mM ddATP/500 μ l TE Buffer.
ddCTP	500 μ l 0.5 mM dTTP+ 25 μ l 0.5 mM dCTP + 500 μ l 0.5 mM dGTP + 10 μ l 10 mM ddCTP/1000 μ l TE Buffer.
ddGTP	500 μ l 0.5 mM dTTP + 500 μ l 0.5 mM dCTP + 25 μ l 0.5 mM dGTP + 18 μ l 10 mM ddGTP/1000 μ l TE Buffer.
ddTTP	25 μ l 0.5 mM dTTP + 500 μ l 0.5 mM dCTP + 500 μ l 0.5 mM dGTP + 50 μ l 10 mM ddTTP/1000 μ l TE Buffer.
Gel-loading buffer	10X: 0.25% bromophenol blue, 0.25% Xylene cyanol and 25% Ficoll (type 400) in H ₂ O.
PBS	Phosphate buffer saline: NaCl 8 g, KCl 0.2 g, KH ₂ PO ₄ 0.2 g and NaHPO ₄ 1.49 g/L pH 7.2.

Sequence Mixtures 1 μ l of (³⁵S) dATP (α S) (400 Ci/m mol) is added to 20 μ l of each of the following

dideoxyribonucleotide triphosphate (ddNTP)

Soln. I (Birnboim) 50 mM glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA.

Soln. II (Birnboim) NaOH 0.2 N, SDS 1%.

Soln. III (Birnboim) 5M Calcium acetate pH 4.8: 60 ml 5M calcium acetate, 11.5 ml glacial acetic acid and 28.5 ml H₂O.

10xTBE 108 g Tris, 55 g boric acid, 9.3 g EDTA/1L pH 8.

0.5 TBE-MIX 150 ml (40%) acrylamide, 50 ml (10xTBE), 460 g urea/1L.

2.5-TBE-MIX 150 ml (40%) acrylamide, 250 ml (10xTBE), 460 g urea, 50 g sucrose, 50 mg bromophenol blue/1L.

TE Buffer 10 mM Tris-HCl pH 8, 1 mM EDTA.

TM Buffer 100 mM Tris pH 8, 50 mM MgCl₂.

Tris buffer (1 M) 121.1 g Tris, 800 ml H₂O and 40 ml HCl/1L pH 8.1.

Bacteria and media:

E.coli K-12 CMK (F⁺, hsdR⁻_k, hsdM⁺_k, lac Y, thr⁻, leu⁻, thi⁻ tonA, supE, lambda^S)

E.coli K-12 SFX (F⁻, hsdR⁻_k, hsdM⁻_k, recB⁻, recC⁻, lop-11, tonA1, thr-1, leu-1, thi-1 supE44, lacY, sbc⁺).

E.coli K-12 SFX recA⁻ (cf.SFX and recA56).

E.coli C-1 G. Bertani, chromosomal marker: none, by definition

TBY-Medium 10 g bacto-tryptone, 5 g yeast extract, 5 g NaCl/1L H₂O.

PAM-Medium 10 g Casmino acids, 10 g nutrient broth, 10 g glucose and 100 g sucrose. after autoclaving, 0.2 g MgSO₄ is added.

Top agar 7 g agar and 8 g nutrient broth/L H₂O.

Bottom agar 5 g yeast extract, 10 g tryptone, 10 agar and 12 g glucose/L H₂O.

BACTERIAL STRAINS:

The E.coli-K12 strains used in our laboratory were provided as follows: CMK, was kindly provided by H.Gram, Ruhr-Universität Bochum and SFX and SFX recA⁻ by W. Wackernagel, Universität Oldenburg. E.coli C-1 G-Bertani was kindly provided by Dr. Bachmann, USA.

ENZYMES AND CHEMICALS:

The restriction enzymes BamHI, EcoRI, EcoRV, NruI, PvuI, PvuII, SalI and ScaI were purchased from Boehringer Mannheim, NciI, PstI, SstII, StuI and XhoI from Bethesda Research Laboratories (BRL), SacII and SinI were purchased from Promiga Biotic, Tth111I from Pharmacia. RNase A, Klenow Fragment of DNA polymerase I, unlabeled deoxyribonucleoside triphosphates and SalI oligonucleotide primer were from Boehringer, Mannheim. Dideoxyribonucleoside triphosphates came from PL Biochemicals and (³⁵S) dATP (α S)(400 Ci/m mol) from Amersham-Buchler. Agarose and TEMED were from Sigma Chemical Co., acrylamide, N,N'-bisacrylamide from Serva, Heidelberg and bind-silane from Bormma, Sweden

PLASMID AND VIRAL DNA:

pBR322 DNA was isolated from the transformed CMK (see below) or purchased from Boehringer Mannheim, ϕ X174 DNA (ss and ds forms) was from BRL.

II-EXPERIMENTAL PROCEDURES

RESTRICTION DIGEST:

To 1-5 μ g DNA in the recommended buffer (according to the producer), 1-35 units of the enzyme were added then incubated for one hour at 37°C. Preliminary tests were performed to evaluate the suitable enzyme units and incubation time which give complete linearization of the DNA used. The reaction was terminated by heating the mixture for 10 min at 65°C (for Tth111I, the reaction was carried out at 65°C). The linearized plasmid or ϕ X174 DNA were separated by agarose gel electrophoresis. The linear DNA fraction was extracted from gel

slices by the electro-elution technique (Maniatis et al., 1982). The DNA was concentrated by ethanol precipitation and stored in TE buffer at -20°C according to Maniatis et al., (1982).

TRANSFORMATION OF PBR322:

Cell culture and competent cells:

Cells were grown in TBY medium (one colony in 5 ml) for overnight (overnight culture). The cells (0.5 ml) were then transferred into 200 ml of TBY medium and grown again at 37°C with shaking to reach $O.D_{590} = 0.4$ (cell number of about 1×10^8 /ml). The cells were held on ice for 15 min then sedimented by centrifugation at 7000 rpm for 10 min at 4°C. The cell pellet was washed in 100 ml cold NaCl (10mM), sedimented and then resuspended in 100 ml cold $CaCl_2$ (30 mM) and kept on ice for 15 min. The cells were sedimented as before and resuspended in 10 ml of the cold $CaCl_2$ (30 mM), glycerol (15% v/v) was added and the cells were then divided into 230 μ l portions (in small Eppendorf plastic tubes) and stored at -70°C until transformation was performed. The transformation technique employed was that of Cohen et al., (1972) slightly modified by Bien (1986).

Antibiotic plates:

The agar plates contained 1.5% (w/v) agar. For the antibiotic plate preparation, media were first supplemented with 100 μ g/ml ampicillin or 10 μ g/ml tetracycline before pouring the media.

Assay of biological activity:

The frozen competent cells were thawed on an ice bath and to each Eppendorf tube (about 2×10^9 cells/230 μ l), 100 μ l $CaCl_2$ (30mM) and 1-10 μ l plasmid pBR322 DNA (100-300 ng) were added. The mixture was held on ice for 30 min then a heat-shock was applied for 5 min at 42°C. The samples were brought to room temperature, and 2.7 ml TBY were added and an expression period was followed for 60 min at 37°C. The probes were then kept on ice until plating was finished. From the transformed cell-

mixture obtained, 30-300 μ l were plated onto the antibiotic plates (dilutions were prepared to give 30-300 colonies per plate). The plates were then kept for 16-20 hrs (or more according to the type of experiment needed) at 37°C and the colonies formed were counted.

ANTIBIOTIC-SENSITIVITY SELECTION:

The linearizing of pBR322 at the tetracycline gene was carried out by digestion with enzymes BamHI, SalI, or NruI and the transformants were selected by plating on ampicillin-agar. PstI, PvuI, or ScaI cut the plasmid in the ampicillin gene, and transformants were selected by plating on tetracycline-agar. For plasmid linearized with PvuII or Tth111I, which cut the plasmid far from the two antibiotic genes (between the Tet. gene and the origin of replication), the transformants were selected by plating on ampicillin-agar. The antibiotic selection of clones was performed by single colony re-examining on new ampicillin or tetracycline plates by the toothpick technique (using sterilized toothpicks). To study the behavior of clone-sensitivity to the antibiotic during incubation time, random clones (200-300) were re-examined directly after their appearance ($\approx$ 18 hr) by transforming to new plates containing ampicillin or tetracycline and then incubated overnight at 37°C. The clones formed were resistant to the antibiotic used while those which did not form, were sensitive. This technique was repeated for different times after the appearance of miniclones (the clones were taken from the original plates which were still incubated at 37°C).

CLONE ANALYSIS:

The clones were separately analysed for their pBR322 DNA plasmid contents according to Birnboim and Doly, (1979) and Maniatis et al., (1982). In this method a single clone was transferred to 5 ml TBV media containing antibiotic (100 μ g/ml amp. or 10 μ g/ml tet.) and incubated overnight at 37°C. From the grown cell-suspension, 1.5 ml was transferred to clean Eppendorf tube for plasmid extraction. The tube was centrifuged for 15 min at room temperature at about 11000 rpm (using an

Eppendorf centrifuge) and the pellet was removed carefully and resuspended in 100 μ l of Solution I and kept at 0°C for 30 min. After incubation, 200 μ l of Solution II were added and the contents were gently mixed and kept at 0°C for 5 min then 150 μ l of Solution III were added. The tube was gently shaken by inversion for a few seconds, during that time a DNA pellet was formed. The contents were allowed to stand for 60 min at 0°C, and then were separated by centrifugation for 5 min (to separate the proteins, high molecular weight RNA and chromosomal DNA). From the clear supernatant, 400 μ l were transferred to another clean Eppendorf tube and 1 ml of cold ethanol was added and the solution was then held at room temperature for 30 min. The precipitated DNA was collected by centrifugation (5 min) and after the removal of the supernatant, the pellet was suspended in 100 μ l of 0.1 M sodium acetate /0.05 M Tris-HCl pH 8. The DNA was precipitated by ethanol (2 volume), centrifuged and then dissolved in 50 μ l Tris-buffer as before.

DNA IRRADIATION:

Irradiation was carried out with a ^{60}Co - γ -ray source (Nuclear Engineering Ltd., Great Britian) at room temperature ($\approx 20^\circ\text{C}$) under air. ϕX174 DNA samples (3.3 $\mu\text{g/ml}$) were first dialysed against phosphate (PBS) buffer (0.01M, pH 7.2) to reduce the scavenging effect of Tris buffer present in the commercial sample. The dialysis was performed in dialysis set (type 1200 MA from BRL) for about 4 hr at 4°C. The dose rate used was 1.17 Gy/min for both ss and ds DNA forms and was measured with a the Frike dosimeter.

TRANSFECTION OF ϕX174 DNA:

Spheroplast-preparation:

Bacterial spheroplasts were prepared according to Guthrie and Sinsheimer (1963) with some modifications (TBY media was used instead of the authors media). *E. coli* K-12 AB1157 cells were grown at 37°C in TBY with aeration until 5×10^8 cells/ml. From the cell suspension, 20 ml were centrifuged at 1000 g for 5 min and the pellet was taken up in 0.35 ml sucrose (1.5 M).

The following solutions were then added in order, with gentle mixing after each: 0.17 ml (30%) bovine serum albumin (BSA), 0.02 ml (2 mg/ml of 0.25 M Tris buffer pH 8.1) lysozyme, 0.04 ml EDTA (4%). After 1 minute incubation with enzyme, 10 ml of PAM medium were added. The contents were incubated for 10-15 min at room temperature, then 0.2 ml MgSO_4 (10%) were added to complex the EDTA and stop the enzyme reaction.

Assay of biological activity:

The biological activity of the two forms of viral DNA was measured according to Blok *et al.*, (1967) with some modifications. Immediately after irradiation, the samples were diluted with PBS buffer to reach 0.1-0.5 $\mu\text{g/ml}$ (for concentration up to 0.5 $\mu\text{g/ml}$ the yield of the biological test is proportional to DNA concentration). Of the DNA solution to be tested, 0.1 ml was incubated with 0.1 ml of spheroplast-suspension for 15 min at 37°C. To the mixture, 0.8 ml of pre-warmed PAM medium was added and the incubation was continued for 90-120 min. Then 9 ml of TBV were added (to open any unlysed spheroplasts by osmotic shock). So as to measure the infective centers, the samples were subdiluted to give 0.3-300 pg DNA per plate). From the diluted samples, 100 μl was mixed with 200 μl plating bacteria (*E.coli* C-1, freshly grown in TBV medium until the log phase at 37°C) and then kept for 10 min at room temperature (adsorption period). The contents were then pipetted with 3 ml warmed top agar onto the plates and gently distributed on the plate surface. The plates were incubated overnight at 37°C after which the plaques were counted. For the transfection of the linear ϕX174 DNA produced by restriction endonucleases, higher concentrations than those used with the irradiated DNA were used (10-50 times) for incubation with bacterial spheroplasts.

AGAROSE GEL ELECTROPHORESIS:

The electrophoretic analysis was performed according to Maniatis *et al.*, (1982) with a Pharmacia apparatus (type GNA-100) and a Shandon Vakam 400 power supply (at 40 mA). The DNA samples were loaded onto 0.9% agarose and electrophoresed for 3

hrs using TBE as buffer (pH 8). The gel was immersed in the electrophoresis buffer containing ethidium bromide (0.5 µg/ml) for about 45 min. The gel was then photographed under a UV lamp (Bachofer, model D-7410 Reutlingen) using a polaroid land film (type 665 photograph).

RECOVERY OF DNA FROM AGAROSE GEL:

For the separation of the DNA from the gel, the area containing the DNA fraction (localized by staining only one lane) was sliced directly after electrophoresis. The slices containing the DNA were placed in a fluid-filled dialysis bag. Most of the buffer was removed carefully from the bag leaving just enough fluid to keep the gel slice in constant contact with the electrophoresis buffer. Just above the gel slice the bag was tied, and immersed in a shallow layer of 0.5X TBE in an electroelution tank. DNA was electroeluted out of the gel and onto the inner wall of the dialysis bag for 3-5 hr (Maniatis et al., 1982). The polarity of the current was reversed for 2 min to release the DNA from the wall of the bag. The dialysis bag was then opened and all the buffer surrounding the gel slice was recovered. The bag was washed out with small quantity of the same buffer. DNA was purified by direct extraction with phenol, phenol-chloroform and chloroform then precipitated and concentrated by ethanol (Maniatis et al., 1982) as mentioned before.

MICROBIOLOGICAL TESTS:

To study whether the miniclones belong to an E.coli mutant strain, several tests have been performed as follows:

1) B-D-glucuronidase and tryptophanase test:

This test was developed by Merck and called Bactident E.coli and consisted of 4-Methylumbelliferyl-β-D-glucuronide, buffering agents. It is prepared in a special kit for rapid identification of E.coli). The activity detection of these enzymes is a specific marker for E.coli (detection was applied according to Diagnostica MERCK).

2) Triple Sugar Iron Agar test:

The appearance of a yellow coloured agar indicates that the

grown strain is of the Enterobacteriaceae (includes E.coli).

3) Christensen-modified Citrate-Agar test:

The positive citrate-result indicates that the bacteria cells are E.coli or one type of Salmonella.

4) DEV Simmon Citrate-Agar test:

This test is more specific for E.coli than test 3.

5) Vogel-Johnson Agar test:

The test was specific to Staphylococcus aureus and Staph. epidermidis but not for E.coli.

6) Leifson-Agar test:

This test is specific for the Salmonella and Shigelle but not for E.coli. The tests 2,3,4,5, and 6 were used as given in Merck Nährböden Handbuch, (1986).

ISOLATION OF THE CCC PLASMID DNA:

pBR322 plasmid DNA was prepared from the amp. and tet. resistant clones according to Birnboim and Doly (1979). A selected colony from the plasmid transformed cells was grown overnight in 20 ml TBV medium containing ampicillin (100 µg/ml) at 37°C. The overnight culture was inoculated to 500 ml TBV medium and grown by shaking at 37°C until O.D₅₉₀ reached about 0.6. The bacteria cells were pelleted at 7000 rpm, for 10 min and resuspended in 6 ml of solution (I), then 1 ml lysozyme solution (14 mg/ml) was added and the contents were incubated for 30 min at room temperature. Then 14 ml of solution (II) were added and the contents were gently mixed and maintained on ice bath for 10 min after which 10.5 ml of solution (III) were added. The contents were again gently mixed by inversion for a few seconds, during which time a pellet of DNA was formed. The tube containing the pellet was maintained at 0°C for 60 min. The denatured protein, the high molecular weight RNA and chromosomal DNA were pelleted by centrifugation at 20,000 rpm for 20 min (at 0°C). The supernatant was then transferred to a siliconized corex-tube and precipitated with 2/3 volume of isopropanol. The solution was held for 15 min at room temperature and then centrifuged at 1000 rpm for 30 min. The precipitate obtained was washed with 5 ml 80% ethanol and dried

under vacuum. The dried material was dissolved in 8 ml TE buffer pH 8 and kept overnight. The pBR322 plasmid DNA fraction was separated either by CsCl-EtBr density gradient centrifugation or by gel electrophoresis technique following the method of Maniatis et al., (1982). In our hand, the latter technique was better for the separation of the supercoiled (ccc) form of the plasmid free from RNA, other DNA forms and the nucleoproteins. The supercoiled form was separated by agarose gel electrophoresis using 1% agarose in TBE buffer (pH 8.0) as mentioned before. The area containing the ccc-form plasmid was sliced carefully and the DNA was extracted from gel by the electroelution technique for about 2 hr. The DNA was dissolved in 1-2 ml TE buffer and purified by phenol-chloroform extraction then concentrated by ethanol precipitation and stored in TE buffer at -20°C until used (Maniatis et al., 1982). RNA could be removed from the sample by the addition of RNase or by separation of DNA by gel electrophoresis.

RESTRICTION OF THE DNA AND DELETANTS PREPARATIONS:

About 1-5 µg of prepared ccc pBR322 DNA was linearized by complete digestion with an appropriate amount of BamHI (1 unit/µg) in the recommended enzyme-buffer for 1 hr at 37°C. The reaction was terminated by heating to 65°C for 10 min. The linearized DNA was subjected to preparative electrophoresis as mentioned above. The linear DNA bands were isolated as in ccc-form. A suitable amount of linear plasmid DNA (100-300 µg) was transformed to CMK bacteria. From the produced clones, a selection of transformants sensitive to tetracycline was made by plating out some clones (ampicillin resistant, Amp^r) on nutrient agar plates containing tetracycline (10 µg/ml). The selected clones which were ampicillin resistant and tetracycline sensitive (Amp^r- Tet^s) were then grown in TB media containing ampicillin. The DNA was separated from these clones by the same technique used for the normal plasmid.

SEQUENCING PROTOCOL:

a) Restriction analysis:

Deletion end-points of deletants were determined by the dideoxy chain termination method (Sanger et al., 1977) with the modification of Kang and Wu, (1987). The extent of the deletion for each mutant (deletant) was first approximated by restriction analysis. The following restriction endonucleases were tested:

EcoRI, EcoRV, BamHI, SalI, NruI and AvaI, and the condition for restriction by each enzyme was employed by using the corresponding buffer. From the restriction analysis, we evaluated the approximate length of each deletion, which was helpful in choosing the suitable primer for each deletant.

b) Primers:

The SalI oligonucleotide primer from Boehringer was used for the sequencing of control DNA (ccc form). Two oligonucleotide primers complementary to the deletant plasmids at positions adjacent to two of the unique restriction endonuclease cleavage sites (EcoRI and EcoRV) have been chemically synthesized. The synthesized primers were purified by using HPLC technique (see Appendix). The sequences of the primers were designed using the sequence of plasmid DNA as determined by Sutcliffe, (1979) and modified by Peden, (1983). The oligonucleotides are 20 bases long and initiate synthesis from the first nucleotide upstream of the respective endonuclease cleavage site. The primer sequences are as follow:

EcoRI primer: 5'-GAATTCATGTTTGACAGC-3'

EcoRV primer: 5'-GATATCGTCCATTCCGACAG-3'

SalI primer: 5'-ATGCAGGAGTCGCAT-3' (commercial)

c) Technique:

- 1) In 1.5-ml eppendorf tubes, ca 1 µg of the isolated ccc-plasmid DNA was combined with 10 µl 0.4 N NaOH and incubated at room temperature for 5 min.
- 2) After the incubation the following solutions were added

without incubation in the following order: 1-3 μ l sequencing primer (0,6-6 ng or 0.1-1 pM), 3 μ l sodium acetate (3 M) pH 4.8, and the volume was made up to 20 μ l with water, then 3 volumes of ethanol (96%) were added. The hybrid DNA was held for 20 min at -70°C and centrifuged at 11,000 rpm for 10 min. The pellet was then washed with 1 ml 80% ethanol and reprecipitated by centrifugation as before. The contents were dried under vacuum then resuspended in 1 μ l TM buffer and 9 μ l water.

- 3) In four newly cleaned eppendorf-reaction tubes (marked with the letters T, C, G and A) 2 μ l of the primer-plasmid and 2 μ l of the radioactive (α S)dATP-sequencing mixture (labelled dATP mixed with dideoxyribonucleoside) were added to the corresponding tube. The reaction was started by the addition of 2 μ l DNA-polymerase I klenow fragment (0.1 unit/ml) for each tube. To ensure that the contents were mixed, the tubes were centrifuged for a few seconds in the eppendorf-centrifuge. The contents were incubated at room temperature for 15 min and a chase of 2 μ l of 0.25 mM dNTPs were added to each tube in the same manner. After 15 min, reactions were stopped by addition of 4 μ l of formamide dye mixture to each tube. The reaction tubes were placed in boiling water bath for 3 min just prior to loading 2 μ l of the samples onto the gels.

d) Gels and electrophoresis:

Gels were set between 20 cm x 40 cm glass plates separated by 0.4 cm Plasticard strips. The loading wells were 3 mm wide and set 1.5 mm apart and were formed by using Plasticard combs. The buffer gradients-gels technique of Biggin et al., (1983) was used in this study. The sequence gel mixtures were prepared according to the following scheme :

Mixture I: 25 ml, 0.5X TBE-MIX. + 125 μ l APS (100 $\mu\text{g/ml}$) + 50 μ l TEMED.

Mixture II: 7 ml, 2.5X TBE-MIX. +35 μ l APS (100 $\mu\text{g/ml}$)+ 14 μ l TEMED.

The polymerization of acrylamide was initiated by addition of ammonium persulphate solution (APS) and N,N,N',N'-Tetramethyl ethylenediamine (TEMED) to the TBE mixture. Directly after addition of the polymerizing agents, the gradient gel was poured in the following manner. Mix I then Mix II were drawn into a suitable pipette. As judged by bromophenol blue, there was a two-phase solution separated by a diffuse boundary. This was mixed slightly by causing one or two bubbles to run up the pipette by using a short sucking action of the pro-pipette, and then was poured in the gel form. The gel takes about 30 min to polymerize. The gel was then monitored by electrophoresis with 0.5X TBE buffer in the upper and 1X TBE buffer in the lower chamber. The gel was run at ca 1900 V and 38 W for about 2-3 hr. After separation of the plates, the gel was fixed in 1 liter of 10% acetic acid solution and 10% methanol (v/v) for 10-15 min. The gel was then transferred to Whatman 3 MM chromatography paper, covered with Saran Wrap and dried down for 1 hr on a gel drier at 80°C. The Wrap was removed and the gel was then autoradiographed overnight.

CHAPTER 3

COMPARISON OF BIOLOGICAL ACTIVITY FOR ϕ X174 DNA DAMAGED BY
 γ -IRRADIATION OR BY RESTRICTION ENDONUCLEASES

SUMMARY

Single-stranded or double-stranded ϕ X174 DNA was irradiated with γ -rays in aqueous solution under air at room temperature and then transfected to E.coli spheroplasts. Results showed that the dose responsible for biological inactivation of dsDNA was about 6-times higher than that for the ssDNA form ($D_{37} \approx 6$ and 1 Gy respectively). The inactivation was drastically reduced by the addition of t-butanol as scavenger, and this reduction did not reach saturation even at high concentrations used (1.5 M).

Double-strand breaks (dsb) were obtained by the action of different restriction endonucleases on the dsDNA form and the biological activity of the linear DNAs thus produced was studied. The majority of the linear DNA molecules were biologically inhibited and only few were re-cyclized in the cells ($\approx 1\%$) after transfection. In general, the linear ds ϕ X174 DNA with cohesive ends gave higher biological activity than that with blunt ends. However, the length of the overlapping of the cohesive ends does not play an important role. The results were comparable to those obtained from linearized pBR322 DNA by restriction enzymes with respect to the type of restricted ends and the site of dsb produced. A comparison of the effect of γ -rays and endonucleases showed that a 22-24 Gy dose was required to reduce biological activity to 1-2%. This effect was equal to the action of restricted endonucleases when used for the complete linearization of dsDNA form. However, the linear DNA molecules produced by γ -irradiation showed higher biological activity than that produced by restriction endonucleases (≈ 8 times). This may be due to the longer overlapping of the ends produced by irradiation as well as to the random distribution of the dsb

produced. The latter will raise the probability of the homology sequence needed for the recombination processes of linear DNA molecules.

INTRODUCTION

The bacteriophage ϕ X174 DNA was a suitable subject for the study of the comparison of the effects of ionizing radiation, chemical treatment and restriction with enzymes. The advantage is that the DNA can be used in single-stranded or double stranded (ss and ds) forms and possesses biological activity which can be measured simply and quantitatively. In DNA exposed to ionizing radiation, different types of damage can be distinguished, c.f single-strand breaks (ssb), double strand breaks (dsb), cross-links or nucleotide damage (in base or sugar). The specific endonucleases which act on a unique position on the DNA molecule give only one dsb resulting in linear DNA. The contribution of ssb and dsb to the inactivation of bacteriophages has been extensively investigated (Van der Schans and Blok, 1970; Van der Schans et al., 1973). The chemical nature of strand breaks in both ss and ds ϕ X174 DNA has also been studied (Lafleur, et al., 1976 and 1978; Nabben et al., 1984 and Lafleur and Loman, 1986). ssb are a potentially lethal damage for the ssDNA but not for the dsDNA while dsb are lethal for the dsDNA (Jansz and Pouwels, 1965, Van der Schans et al., 1973 and Schulte-Frohlinde, 1987). However, 1 ssb out of ≈ 20 leads to deactivation of the dsDNA. The authors showed that the enzymatically produced dsb are a lethal form of damage comparable to dsb induced by irradiation but it is not 100% lethal. More comparative explanation concerning the high-energy irradiation damage on both ss and dsDNA in connection with the damage produced by restriction endonucleases, is the goal of this study. The biological consequences of strand breakage produced by γ -rays in biologically active ss and ds ϕ X174 DNA were studied. The comparison was also made with dsb produced by

different restriction-endonucleases. The data obtained was also compared to that for the pBR322 plasmid DNA treated in an analogous manner in the present study.

RESULTS

EFFECT OF γ -RAYS ON ϕ X174 DNA:

Survival of ss and dsDNA:

Irradiated ssDNA molecules (3,3 μ g/ml) under aerobic conditions were transfected into E.coli spheroplasts. The number of phage plaques decreased linearly with increasing dose and the D_{37} was about 1 Gy (Figure 1). However, the irradiation of viral dsDNA form under the same conditions only slightly affected the biological activity of the DNA. At higher doses the effect on the biological activity was clear and the D_{37} value was about 6 Gy. From these values (Figure 1), follows that the dsDNA molecules need about 6-times higher doses than ssDNA to reach the same level of inactivation (6.6-times a value was given by Lafleur and Loman, 1986).

Effect of t-Butanol:

The t-butanol was added at different concentrations to dsDNA solution before irradiation to evaluate its scavenging efficiency for water radicals produced by γ -rays. Its addition strongly reduced the biological inactivation in the irradiated viral DNA as shown in Figure (2). The data obtained showed that by increasing the t-butanol concentration (0.01-1 M) the inactivation was decreased linearly. However, at higher alcohol concentrations (1.5 M) the rate of reduction was lower (but not reached a saturation point, Figure 3).

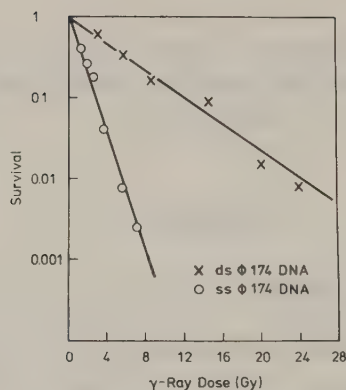


Figure 1: Survival of ss and ds ϕ X174 DNA irradiated in phosphate buffer (0.1 mM, pH 7.2). The applied dose rate was 1.17 Gy/min.

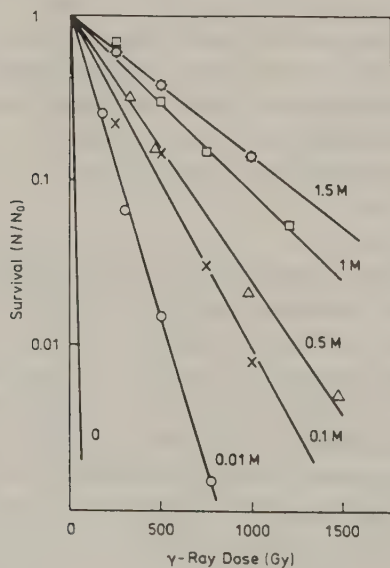


Figure 2: Survivals of 3.3 μ g/ml ϕ X174 DNA (ds) irradiated in 10 mM phosphate buffer pH 7.2 with different concentrations of t-butanol as indicated.

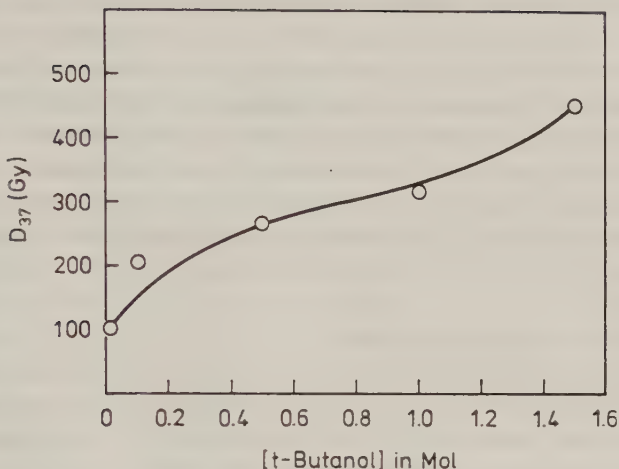


Figure 3: Effect of the concentration of t-butanol as scavenger on the D_{37} value of ds ϕ X174 DNA samples (3.3 μ g/ml) in phosphate buffer (10 mM, pH 7.2).

The effect of γ -irradiation is considerably affected by DNA concentration, buffer, oxygen content and the presence of salts as well as the presence of scavenger (Blok et al., 1967). In an additional experiment, the irradiation conditions first used were altered as follows: 1.1 μ g/ml dsDNA, purging by N_2 , disodium tetraborate buffer (1 mM, pH 9.2) and addition of $MgCl_2$ (1 mM). The effect of irradiation was 5-times higher than that obtained in the previous conditions (comparison was done for a t-butanol concentration of 0.5 M). The same result was reported by Nabben et al., (1984) under the same conditions.

It is appropriate to mention that the presence of phosphate ions in the buffer used do not have a significant

effect on the damage of ϕ X174 DNA by γ -rays under the aerobic conditions typically used in the present work. This was already observed by Achey and Duryea, (1974) and Lafleur, et al., (1975). The authors reported that the phosphate ions of the buffer had no effect on DNA during and (after) γ -irradiation under aerobic conditions, even when different concentrations were used. Under N_2 saturation, however, the phosphate ions have a sensitizing effect by reacting with hydrated electrons. Brustand and Wold, (1976) showed also that under N_2O the phosphate ions could be sensitized (during γ -irradiation) in the presence of NaCl by reaction with Cl ions.

EFFECT OF RESTRICTION-ENDONUCLEASES:

Nine restriction enzymes each producing a unique dsb in the ds ϕ X174 DNA, were used. The enzymes were chosen to act at different positions on the genome and produce different types of restricted ends, blunt or cohesive, with different base extensions. When the linearized DNA molecules were transfected to E.coli spheroplasts, the transfection efficiency was found to have been strongly affected. A very low number of the linear DNA molecules was re-cyclized in the host and produced plaques ($\approx 1\%$) when compared to that produced from the untreated DNA (Table 1). Data in Table (1) shows that the blunt-ended DNA linearized with StuI (ϕ X174 DNA/StuI) gives the lowest biological activity (0.65%) this being about half the transfection efficiency value of the DNA with cohesive ends). The ϕ X174 DNA/AvaII gives the highest value (1.7%) which represents 2.6-fold increase with respect to ϕ X174 DNA/StuI. Although the linear DNAs which contained cohesive ends are different in base-extension, no big differences in biological activities were noticed (Table 1). The results obtained here are similar to those obtained by the restriction enzyme treatment of pBR322 plasmid DNA. However, some types of linear plasmid gave the opposite behaviour in connection with the type of restricted ends (see the restriction of plasmid section).

Table 1. The biological activity of ϕ X174 phage dsDNA linearized by different restriction enzymes as compared with ccc form.

Linear DNA	Base exten.	No. plaques/ μ g DNA		% Biol. activ.	Relative* biol. activ.
		Linear DNA	ccc DNA		
ϕ X DNA/ <u>PstI</u>	4	1.1×10^6	7.7×10^7	1.30	2.00
ϕ X DNA/ <u>AvaI</u>	4	8.7×10^5	7.7×10^7	1.14	1.75
ϕ X DNA/ <u>XhoI</u>	4	8.2×10^5	7.7×10^7	1.07	1.64
ϕ X DNA/ <u>AvaII</u>	3	1.3×10^6	7.7×10^7	1.70	2.61
ϕ X DNA/ <u>SinI</u>	3	8.7×10^5	7.7×10^7	1.12	1.72
ϕ X DNA/ <u>SacII</u>	2	1.0×10^6	7.7×10^7	1.30	2.00
ϕ X DNA/ <u>SstII</u>	2	1.1×10^6	7.7×10^7	1.43	2.20
ϕ X DNA/ <u>NciI</u>	1	2.5×10^6	$1.0 \times 10^8^{**}$	1.50	2.30
ϕ X DNA/ <u>StuI</u>	0	6.5×10^5	$1.0 \times 10^8^{**}$	0.65	1.00

* The values were taken relative to ϕ X DNA/StuI.

** New ϕ X174 DNA sample.

From Table (1) although the used restriction enzymes acted at different positions of the DNA molecule (at different genes), the different types of the produced linear DNAs did not show big difference in the survivals. Therefore, the position of dsb did not play an important role on the repairing efficiency of these breaks (as long as the dsb is far from the origin of replication, Sutcliffe, 1979 and Conley and Saunders, 1984). In addition, the length of the end-overlap is not so important in transfection efficiency as found from the present data. The sequence of these ends, however, may be essential for the repairing processes (as intramolecular recombination as mentioned by Bergasma *et al.*, 1982; Orr-Weaver *et al.*, 1981; Abastado *et al.*, 1987). The sequence of the terminus in turn acts as a recognizer for a suitable homology in the DNA strands which is necessary for the intramolecular recombination processes and which may results in a deletion (reported also by

Albertini et al., 1982; Conley et al., 1986b and Nash et al., 1987).

From the results obtained in Table (1), most of the linear DNA transfected to E.coli spheroplasts were degraded by the host enzymes. The degradation was due to the action of endo- and or exo-nucleases. The surviving phage particles resulted from the re-cyclizing of some (<2%) linear DNA molecules by the bacterial (and phage) enzymes without destruction or loss of the genetic information. Therefore, the re-cyclizing of a linear ϕ X174 DNA is a rare event in bacterial cells and degradation is the rule (as reported for linear plasmid by Symington et al., 1985 and Schulte-Frohlinde, 1987). The repairing of the linear DNA proceed via the intramolecular recombination processes is discussed in detail in the plasmid and sequencing sections.

DISCUSSION

Comparison of γ -ray- and restriction-induced DNA breaks is difficult. The production of double-strand breaks by the latter can be assumed to show similar variabilities as the inhibition of survivals. Further, the molecular mechanisms by which γ -rays or restriction endonucleases produce such breaks are entirely different. The endonucleases can be expected to produce high quantities of double-strand breaks, all of which will have 3'-OH and 5'-phosphate ends, and can easily be sealed by polynucleotide ligases (or other enzymes). Gamma rays produce different types of double-strand breaks, only some of these will have the structure of the ones produced by restriction endonucleases. However, Natarajan and Obe, (1984) observed that response of cells to treatment with restriction endonucleases is very similar to that of ionizing radiations. They showed that the types of chromosomal aberrations (in Chinese hamster ovary cells) induced by restriction endonucleases were also similar to those induced by the ionizing radiations, though the relative proportions of the various types were

different. Similar observation was given by Bryant et al., (1987) who reported that the dsb induced by restriction endonucleases were handled by the cells in a similar way to that for X-ray-induced dsb.

In this respect, it is interesting to compare the effect of irradiation on the viral DNA with the effect of restriction enzymes. From the results obtained in the present work, the dose needed to reach D_{37} for the dsDNA was 6 Gy (Figure 1). From the survival curve, the dose required for 98-99% inactivation (1-2% residual activity) was about 22-24 Gy. This reduction in activity is comparable to that resulting from the action of endonucleases, which digest ds ϕ X174 DNA completely to the linear form (each molecule contains a unique dsb). From the experiments, 1 μ g DNA could be completely linearized with 5-30 enzyme-units according to the activity of the enzyme. Bryant, (1984) gave a similar comparison for mammalian cells either restricted with enzymes which were permeabilized into them or after X-ray irradiation of the cells. He found that treatment with 1 unit of PvuII (blunt-ended) or BamHI (cohesive-ended) had an approximately equivalent effect to 0.11 Gy of X-rays, a dose which have induced more than one aberration per cell. Obe and Johannes, (1987) reported that 2 units of AluI (blunt-ended) or 5.7-7.9 units of BamHI produced the same effect in the chromosome as 1 Gy of X-rays.

It was known that the γ -irradiation induces three types of damage in DNA: ssb, dsb and nucleotide damage (nd) which includes base damage (bd) and damage in the sugar moiety. The latter damage (nd) is not associated with chain breakage or intermolecular cross linkage. The contributions of these three types of damage to inactivation of phage DNA were as follows: $\approx 9\%$, $\approx 4.5\%$ and 87% respectively (Van der Schans et al., 1973). Similar findings were obtained by Van der Schans and Bleichrodt, (1974) when the irradiation was carried out at -196°C ($\approx 5\%$ for ssb, $\approx 10\%$ for dsb and $\approx 85\%$ for nd). By X-rays, the contribution of ssb, dsb, bd (and alkali labile sites) and thymine ring saturation products to inactivation was 4.2, 11,

13.8 and 5.2 % respectively (Moran and Wallace, 1985). From the literature, the number of ssb in relation to dsb is $\approx 10-20$ in the γ -irradiated DNA molecules (Bien et al., 1988), while the number of bd is assumed to be 3-10 times larger than the number of ssb (Ward, 1985). In the present study, the irradiation of ds ϕ X174 DNA with a dose of 23 and 34 Gy gave 1% and 0.1% biological activity respectively (Figure 2). Therefore, the contribution of ssb, dsb and nd ($\approx$ bd) was calculated to be about 7%, 9% and 84% respectively. The lethality of each damage was taken as follows: 16-20 ssb, 1 dsb and 5-10 bd for one lethal event according to Jansz et al., (1968); Schaaper and Loeb, (1981); Van Touw et al., (1985); and Schulte-Frohlinde, (1987). The results obtained by Bien et al., (1988) were very similar to the data presented here, since they found that 30 Gy dose inhibited the biological activity of pBR322 to 0.1%. Van Touw et al., (1985) reported that the D_{37} doses for the introduction of ssb, potential ssb, and dsb, in ds ϕ X174 DNA were 0.42, 1.4, 57 Gy respectively. The induction of 1 dsb per yeast cell was obtained by 18 Gy dose from electron irradiation (Frankenberg et al., 1984).

From the results obtained here, the irradiation of the ds ϕ X174 DNA with low doses (<10 Gy) produced low biological inactivation which was mainly due to the formation of ssb and base damage. Higher doses led to drastically biological inactivation due to dsb formation in addition to high percentages of ssb and bd. As observed from survival curves and electrophoretic separation the linear DNA form represents about 65% of the total DNA sample when irradiated with 34 Gy dose. Then from the previous calculated data dsb contribute $\approx 9\%$ of the deactivation produced by γ -rays. By excluding the effect of ssb and bd damages from the linear DNA molecules (produced by 23 Gy dose), the survival of these molecules is calculated to be $\approx 8\%$. Therefore, the survival of the linear ϕ X174 DNA molecules produced after γ -rays was higher (about 8-times) than that produced by the restriction endonucleases. This may be due to the longer length of overlapping in the cohesive ends produced by irradiation (Bien et al., 1988) and or to the

random distributions of the dsb in the DNA molecule (Webb and Debenham, 1985). The latter will increase the probability of the sequence which can act as matching homology with the end-sequences of the dsb. These factors are essential for the recircularization processes of the linear DNA molecules in the cells (may be by intramolecular recombination).

The major damaging effect of γ -rays on the DNA molecules is due to OH radicals. This radical can be removed to a large extent by the addition of t-butanol, the most specific scavenger for OH radicals. In the present study, the alcohol was used at different concentrations (Figure 2) and the results were consistant with those in the literature. Lafleur et al., 1978 and 1982 reported that during the γ -irradiation of ϕ X174 DNA, t-butanol was a good scavenger for OH radical and did not result in disturbing complications such as secondary reactions of the t-butanol radicals with the DNA. However, in 1986, Lafleur and Loman found that the secondary radicals could be formed from t-butanol by increasing its concentration. But the radicals formed from t-butanol through the reaction with OH radicals were not as effective in inactivating DNA as OH radicals. The scavenging efficiency of t-butanol can be explained by the predicted dependence on the relative yield of the reaction of OH radicals with DNA on the concentration of t-butanol (Van Rijn et al., 1985 and Lafleur and Loman, 1986). This observation is in agreement with the present findings since, by increasing the t-butanol concentration (>1 M) the rate of scavenging was decreased as in Figure 2,3). The alcohol has also been used as protector from γ -rays in cells, where the half concentration for protection was 2.5 M (Roots and Okada, 1972). Furthermore, Ewing and Kubala (1987) showed that the lower concentrations (0.05-0.1 M) of t-butanol gave higher protection for OH radicals and in turn higher survival percentages for E.coli than the higher concentrations (0.3 M). The formation of ssb in native DNA or in DNA models (poly U) by laser pulse can be reduced by high concentrations of ethanol or t-butanol (0.07 and 0.1 M respectively) as reported by Schulte-Frohlinde, (1986). The addition of t-butanol as scavenger for

the OH radical was essential to study the role of OH radical damage as well as the other damages (from H radicals and electrons). Lafleur et al., (1978) were able to obtain damage only from H radicals by irradiation of the dilute ss ϕ X174 DNA solution in 0.01 M phosphate buffer (pH 7.2) with 5 mM t-butanol under nitrogen. Blocking the action of OH radicals by t-butanol is also essential for studying the repairing pathway for each damage. Blok and Verhey (1968) reported that 50% of ss ϕ X174 DNA inactivation is due to the OH radical and 50% to the H radical and solvated electron. One type of damage attributed to the action of the OH radical is the ssb which would constitute a nonsusceptible type of damage since linear ϕ X174 DNA molecules are biologically inactive (>98% as found in the present study). Approximately 10% of the biological inactivation of ds ϕ X174 DNA by γ -rays in the presence of O_2 is due to ssb (Blok and Loman, 1973). If all other forms of damage in the DNA molecules were susceptible to inducible recovery, a percentage efficiency of recovery approaching 90% would be expected. Approximately 50% of the potentially lethal damage produced in both forms of ϕ X174 DNA by H radical and solvated electron are susceptible to inducible repair. These radicals play a small role in the production of ssb (Achey and Durya, 1974). This suggests that the nonsusceptible damage produced by these radicals is something other than ssb, possibly a type of base damage.

Excision and recombination repair are template-directed, and ss ϕ X174 DNA does not possess a template strand to direct the reinsertion of excised nucleotides or the positioning of a homologous strand of DNA (Howard-Flanders, 1973). Also, all known mechanisms of excision repair produce a break in the phosphodiester backbone of the strand which contained the excised damage. Such a break would render ss ϕ X174 DNA biologically inactive (Fiers and Sinsheimer, 1962). Silber and Achey (1984) reported that different irradiation conditions yield damages which vary in their susceptibility to induce repair and that these damages possibly are repaired with equal efficiency in both ss and ds DNA by a common mechanism. The

work of Silber et al., (1979) showed that the damage produced in the γ -irradiated ss and ds DNA can be repaired by the common mechanism based on excision repair, recombinational repair or sb-type repair.

In respect with genes responsible for repairing the damage in ds ϕ X174 DNA by γ -rays, Nabben et al., (1984) showed that the repairing varied according to the type of damage produced and to the gene proficiency in the cell. In the excision DNA repair, part of the radiation damage due to H radicals appeared to be repairable by an *uvrA*-gene-dependent repair mechanism, whereas this repair pathway does not play an important role in the case of OH radical damage. The reverse applies for *uvrC*-gene-dependent repair, which only affects OH radical damage (obtained under anoxic conditions) but has no influence on damage due to H radicals. Furthermore, the post-replication repair (*recA*⁺) has only very little effect on the amount of inactivation by H or OH radicals, when irradiation is carried out under anoxic conditions. The authors added that post-replication or recombination repair appears not to play an important role in repair of damage due to water radicals. This result can be expected for a system in which only one copy of the ϕ X174 molecule is transfected to E.coli spheroplasts.

The repairing of damage produced by the action of restriction endonuclease, however, is to some extent different because the damage are only dsb. In this respect the intramolecular recombination processes are important, producing perfectly repaired or deleted DNA molecules. The enzymes of the bacterial host have a major role in carrying out those processes. These include the polymerases, endo- and exonucleases, ligases, helicases in addition to other cell proteins. Nash et al., (1987) showed that the integration of bacteriophage lambda requires the viral protein *Int* (site specific recombinase) and the host protein *IHF* (integration host factor). While the excision requires an additional viral protein, (*Xis*). Bacteriophage lambda uses genetic recombination both to join its chromosome to that of E.coli and to excise the

intergrated viral DNA. The study showed also that the bacteriophage site (*attP*), the bacterial site (*attB*), and the prophages sites (*attL* and *attR*) all have in common a sequence of 15 bp. The type of the restricted ends and their sequences also have an important role for the recognition of the homologous sequence needed for the recombination processes (see the restriction and sequencing sections).

CHAPTER 4

**BIOLOGICAL DEACTIVATION OF PLASMID PBR322 BY
RESTRICTION ENDONUCLEASES**

SUMMARY

Plasmid pBR322 DNA has been treated with several restriction enzymes to generate double strand breaks (dsb) containing differently structured termini at different positions of the plasmid. The transformation efficiencies of the linear plasmid obtained were studied. In the E.coli K-12 CMK, the yield of transformants produced by the action of the restriction enzymes on plasmid DNA is ranged from 0.1% to 3.3%. In some cases, the blunt-ended linear plasmid gave transformation percentages similar to that of cohesive-ended ones. In this respect, not only is the type of restricted ends of linear plasmid essential for the repairing efficiency, but also the base extension and the base sequence of the terminus. Whether the restricted site is located in one of the different genes or not does not have an important role in the repairing capacity. The majority of the transformants obtained contained perfectly recircularized plasmid DNA molecules while the minority contained plasmid with deleted sequences and showed antibiotic sensitivity. The mutation frequency in the transformants ranged from 0.3-12% according to the type of linear plasmid used for transformation. The antibiotic sensitive mutations showed different deletion lengths in their plasmid DNA (100-2000 bp) depending on the type of the linear plasmid DNA used. The linear plasmid which gave low transformation efficiency produced the more antibiotic-sensitive mutants with longer deletion size and vice versa. The transformation efficiency of linearized plasmid showed a similar behaviour when wild-type SFX or SFX recA^- strains were used for transformation. The effect of blunt-end creation as well as saturation conditions on the transformation efficiency were studied.

INTRODUCTION

When the pBR322 plasmid DNA was linearized and then introduced into bacterial cells, a much lower number of transformants was obtained than that with the corresponding covalently-closed circular DNA ,cccDNA, form (Thompson and Achtam, 1979; Conley and Saunders, 1984 and Bien, 1986). The enzymatically linearized plasmid which is transformed into E.coli cells can be repaired by the enzymes of the host. The repairing was accompanied by the appearance of mutants containing deletions that extended for various distances from the cleavage site that resulted in linearization (Thompson and Achtam, 1979; Garaev et al., 1982 and Conley et al., 1986a,b). The repair of DNA damage [single strand break; double strand break and base damage] has also been demonstrated as a process which is accompanied by the generation of mutations in bacteria and phages (Bridges and Mottershead, 1978; Silber et al., 1979; Fluke and Pollard, 1978 and 1982). The relative proportion of perfectly re-cyclized products to aberrant recircularization products is dependent both on host recombination functions and the nature of the ends of the linear plasmid DNA molecules used as substrates for transformation (Conley et al., 1986a,b and Bien et al., 1988). The formation of dsb on the DNA molecules inside the cells (by restriction enzyme injection) has also been studied (Bonura et al., 1975; Bryant, 1986; Bryant et al., 1987 and Winckler et al., 1988) and similar results have been found.

In these other studies mentioned above, only one or two restriction enzymes were used and explanations were given for the reduction in the biological activity and the effect of end type. So as to clarify the findings obtained, we increased the number of restriction endonucleases used to eight. Each enzyme resulted in different cleavage positions and end types. The position of restriction in connection with the plasmid genes and the base extension of the termini will be discussed. The mutation frequencies and the deletion lengths of the mutants

were also measured. pBR322 DNA was chosen because it is a widely used cloning vector and its entire nucleotide sequence is known, (Sutcliffe, 1979 and Peden, 1983).

RESULTS

TRANSFORMATION WITH LINEARIZED PBR322 DNA:

Linear plasmid DNA showed much lower transformation efficiency than normal DNA. Values ranged from 0.1-3.3% of the cccDNA plasmid and depended on the type of restricted DNA molecule (type of ends and position, Table 2 and 3). The transformation efficiency as a percentage of that of untreated pBR322 was calculated from the transformants produced from 1 µg DNA plasmid to obviate the effects of differences in viability and competence levels obtained with different batch cultures of the same strain. The linear plasmid molecules containing overlapping ends (cohesive) led to a higher survival than blunt-ended DNA molecules. However, some linear plasmids with cohesive ends (e.g pBR322/SalI, 4 base extension) showed a lower biological activity (0.5%), than others molecules with the same base extension (3.34 and 1.03% for pBR322/PstI and pBR322/BamHI respectively). In addition, some linear plasmids with blunt ends (e.g pBR322/NruI) showed higher (0.53%) biological activity than plasmids with cohesive ends (e.g. 0.5, 0.46 and 0.26% for pBR322/SalI, pBR322/Tth111I and pBR322/PvuI respectively). The biological activity of the linear plasmid obtained by PvuI, which gives cohesive ends (but with 2 base extension) was quite low and fell well below other linear plasmids with cohesive ends (e.g. pBR322/BamHI and pBR322/PstI) as well as two with blunt ends (e.g pBR322/NruI and pBR322/ScaI). From Table (3) it can also be seen that the restriction of the plasmid DNA in Amp. gene, Tet. gene or in other zones (as long as the restriction was not in the origin of replication), all gave a reduction in biological activity to below 3.5% of ccc-plasmid and thus the difference in this reduction is not as large as might have been expected.

Table 2: Some informations about the restriction endonucleases used and the types of linear pBR322 DNA ends produced.

Enzyme	Type of ends	Base exten.	Sequence restricted	Sequence of terminus	Gene restricted*
<u>Bam</u> HI	cohesive	4	GGATCC CCTAGG	-G GATCC- -CCTAG G-	Tet. (375)
<u>Sal</u> I	cohesive	4	GTCGAC CAGCTG	-G TCGAC- -CAGCT G-	Tet. (651)
<u>Nru</u> I	blunt	0	TCGCGA AGCGCT	-TCG CGA- -AGC GCT-	Tet. (974)
<u>Pvu</u> II	blunt	0	CAGCTG GTCGAC	-CAG CTG- -GTC GAC-	--- (2068)
<u>Tth</u> III	cohesive	1	ACNNGT TGNNCA	-ACN NNGT- -TGNN NCA-	--- (2222)
<u>Pst</u> I	cohesive	4	CTGCAG GACGTC	-CTGCA G- -G ACGTC-	Amp . (3613)
<u>Pvu</u> I	cohesive	2	CGATCG GCTAGC	-CGAT CG- -GC TAGC-	Amp . (3738)
<u>Sca</u> I	blunt	0	AGTACT TCATGA	-AGT ACT- -TCA TGA-	Amp . (3848)

* The position-number of base at which the restriction was proceeded is givin in parentheses (from the EcoRI site).

Table 3: Transformation efficiencies of different types of linear pBR322 DNA obtained by some restriction endonucleases and compared with normal plasmid*.

Plasmid pBR322 DNA	Mean transformants/ μ g DNA		Transform.
	Linear DNA	Normal DNA	Efficiency %
<u>Plasmid restricted on the Tet. gene:</u>			
<u>BamHI</u> -linearized (4)**	4.7×10^3	4.6×10^5	1.03
<u>SalI</u> -linearized (4)	2.9×10^3	5.8×10^5	0.50
<u>NruI</u> -linearized (0)	3.0×10^3	5.6×10^5	0.53
<u>Plasmid restricted far from genes:</u>			
<u>Tth111I</u> -linearized (1)	3.0×10^3	6.6×10^5	0.46
<u>PvuII</u> -linearized (0)	6.5×10^2	6.6×10^5	0.10
<u>Plasmid restricted on the Amp. gene:</u>			
<u>PstI</u> -linearized (4)	1.8×10^4	5.5×10^5	3.34
<u>PvuI</u> -linearized (2)	1.7×10^3	6.5×10^5	0.26
<u>ScaI</u> -linearized (0)	2.0×10^3	5.9×10^5	0.34

* A mean of at least five experiments.

** The value in parenthesis indicated the base extension of restricted terminus.

EFFECT OF S1 NUCLEASE:

When the base extensions of the cohesive termini were removed by the action of S1 nuclease to create blunt-ended linear DNA, the transformation efficiency of the latter was strongly reduced (Table 4). Although the three types of linear DNA tested have the same base extension (4 b), the extent of reduction in the transformation efficiency after blunt-end creation was quite different (9, 7 and 3-fold for linear pBR322/BamHI, pBR322/SalI and pBR322/PstI respectively). In this respect, the type of ends before and after S1 action may not be the only factor affecting the re-cyclization of the

linear plasmid DNA in the cell, and thus determining the transformation efficiency.

DOSE-RESPONSE OF NORMAL AND LINEAR PBR322 DNA:

To compare the dose-response relationships for normal pBR322 DNA and linear pBR322 obtained by digestion with BamHI and PvuII, the wild type E.coli CMK was used as recipient (Figure 4). From the figure, the dose-response slope for normal plasmid DNA at non-saturating DNA concentration is about 1.2 and saturation occurs at above 0.5 μg DNA per approximately 6×10^5 viable cells. Linearized pBR322 DNA (with cohesive or blunt ends) resulted in a slightly lower (1.0) dose-response slope than that of ccc-plasmid. However, the overall efficiency of transformation was reduced approximately 100-fold and 1000-fold (for BamHI and PvuII respectively) relative to normal DNA (Figure 4 and Table 3). This behaviour illustrates that the linear DNA molecules saturate at higher concentrations than normal DNA molecules.

EFFECT OF THE ANTIBIOTIC MEDIA:

When some types of linear DNA plasmids were plated after transformation to E.coli on ampicillin or tetracycline plates, no large difference in the biological activity was obtained (Table 5). The linear plasmid restricted in the Amp. gene gave slightly lower number of transformants on the ampicillin plates than that grown on the tetracycline ones. The same trend was found when linear DNA molecules restricted on the tetracycline gene were plated on ampicillin or tetracycline plates (lower efficiency on the tet. plates).

Table 4: Effect of S1 Nuclease on the biological activity of some types of linear DNA with cohesive ends.

Linear DNA	<u>Transformation Efficiency %</u>		Fold reduction
	before S1	after S1	
pBR322/ <u>BamHI</u>	1.03	0.097	9
pBR322/ <u>SalI</u>	0.50	0.070	7
pBR322/ <u>PstI</u>	3.30	1.100	3

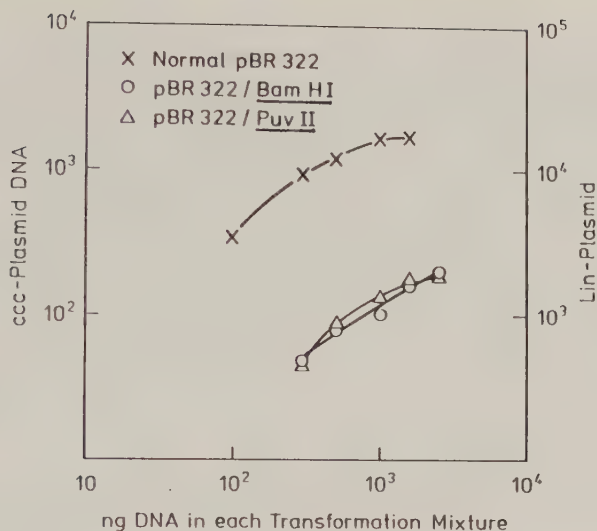


Figure 4: Transformation efficiency as affected by DNA dose. Comparison between ccc and linear form transformed to *E. coli*

Table 5: Effect of antibiotic media on the transformation efficiency of plasmid DNA linearized by some restriction enzymes.

Linear DNA	Gene restricted	<u>Transformation Efficiency %</u>	
		amp. agar	tet. agar
pBR322/ <u>BamHI</u>	Tet.	1.03	1.00
pBR322/ <u>SalI</u>	Tet.	0.36	0.33
pBR322/ <u>PstI</u>	Amp.	2.93	3.34
pBR322/ <u>PvuI</u>	Amp.	0.21	0.26
pBR322/ <u>ScaI</u>	Amp.	0.34	0.40

Table 6: Transformation of E.coli K-12 derivatives with normal and linear pBR322 DNA plasmid.

Plasmid DNA	<u>Transformants/ μg DNA</u>					
	<u>(SFX)</u>			<u>(SFX-recA⁻)</u>		<u>%</u>
	Lin/DNA	scDNA	% Activ.	Lin/DNA	scDNA	Activ.
pBR322/ <u>Bam</u> HI	6.7x10 ³	6.9x10 ⁵	1.00	5.7x10 ³	5.5x10 ⁵	1.04
pBR322/ <u>Pvu</u> II	7.5x10 ²		0.11	9.3x10 ²		0.17
pBR322/ <u>Pst</u> I	2.6x10 ⁴		3.80	1.9x10 ⁴		3.61

EFFECT OF E.COLI K-12 DERIVATIVES:

In addition to E.coli CMK, the E.coli SFX and E.coli SFX-recA⁻ strains were used for transformations of linear DNA molecules and the results are given in Table (6). Although, the SFX-recA⁺ gave higher transformation efficiency for both normal and linear plasmid DNA molecules than CMK, the percentage of transformation efficiency of linear molecules relative to covalently closed circular molecules was similar to that found in CMK strains. In the SFX-recA⁻ strains, the transformation efficiency for both ccc and linear plasmid DNA was very similar to that obtained in CMK strains. From these results it appears that the recA does not play an important role on the repairing of linear plasmid DNA in bacterial cells as will be discussed latter.

MUTANT (DELETION) FREQUENCIES:

It was found that during the repair of linear plasmid DNA probes mutations occur. The transformation of linear plasmid DNA restricted in tetracycline gene showed some mutations which were sensitive to tetracycline but not to ampicillin (Amp^r-Tet^S). On the other hand, the transformation of linear plasmid DNA restricted in ampicillin gene showed some mutations sensitive only to ampicillin (Amp^S-Tet^r). The mutation and in turn the sensitivity to the antibiotic was due to a deletion in the restricted zone during the re-cyclization processes of the

linear DNA molecules in the bacterial cells. Data in Table (7) shows the mutation frequency of the mutants produced by transformation of different types of linear DNA plasmids. From the table, the highest mutation frequency was produced by the transformation of linear pBR322/PvuI followed by pBR322/PvuII and pBR322/SalI (12, 10.3 and 9.7% respectively). The lowest values were found for transformation of linear pBR322/PstI (0.3%) and pBR322/ScaI (1.3%) respectively.

Table 7: Gross mutation frequencies as determined by antibiotic sensitivity of transformants*.

Plasmid DNA	<u>Ampicillin sensitivity</u>		<u>Tetracycline sensitivity</u>	
	Amp ^r %	Del. length	Tet ^r %	Del. length
pBR322/ <u>BamHI</u>	0	----	2.2	200-1250
pBR322/ <u>PvuII</u>	0	----	10.3	900-2010
pBR322/ <u>SalI</u>	0	----	9.7	70-2200**
pBR322/ <u>PstI</u>	0.3	250-650	0	----
pBR322/ <u>ScaI</u>	1.3	160-830	0	----
BR322/ <u>PvuI</u>	12	(***)	0	----
pBR322/ <u>NruI</u>	0	----	4	(***)
pBR322/ <u>Tth111I</u>	0	----	4.3	(***)

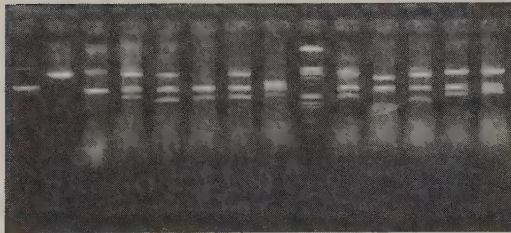
* Deletion lengths (bp) were included.

** Measured by Mrs. M. Merz in our laboratory.

(***) Not measured

For the deletion length-measurments, plasmid DNA was isolated from 20 selected deleted Amp^r-Tet^s (for BamHI and PvuII) or Amp^s-Tet^r (for PstI and ScaI) transformants. The isolated DNA was digested with 2 appropriate restriction enzymes and then analyzed by gel electrophoresis (see Materials and methods and Figure 5). The molecular weights of the restricted DNA fragments were calculated by using the DNA molecular weights standard III as a reference (Figure 5a-d). The size of deletion averages for the tested clones are given in Table (7).

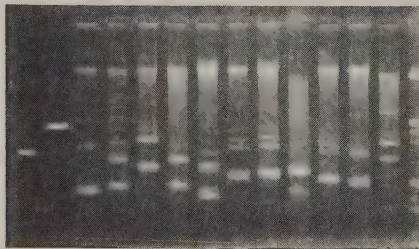
1 2 3 4 5 6 7 8 9 10 11 12 13 14



(a) pBR322/BamHI
clones:

Lane 1: purchased pBR322 DNA Lane 2: linear pBR322/BamHI
Lanes 3-8 and 10-14: deleted plasmids isolated from different
Amp^r-Tet^s clones and digested by EcoRI+BamHI.
Lane 9: DNA molecular weights standard III.

1 2 3 4 5 6 7 8 9 10 11 12 13 14



(b) pBR322/PvuII
clones:

Lane 1: purchased pBR322 DNA Lane 2: linear pBR322/PvuII
Lanes 3-13: deleted plasmids isolated from different Amp^r-Tet^s
clones and digested by EcoRI+PstI.
Lane 14: DNA molecular weights standards III.

Figure 5: Agarose gel electrophoresis of deleted plasmids isolated from different antibiotic sensitive transformants and treated with two restriction enzymes: (a) pBR322/BamHI clones (b) pBR322/PvuII clones.

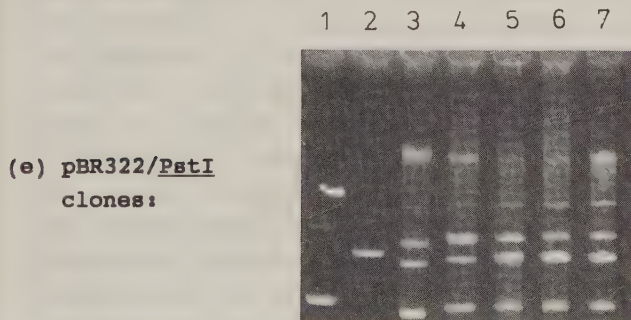
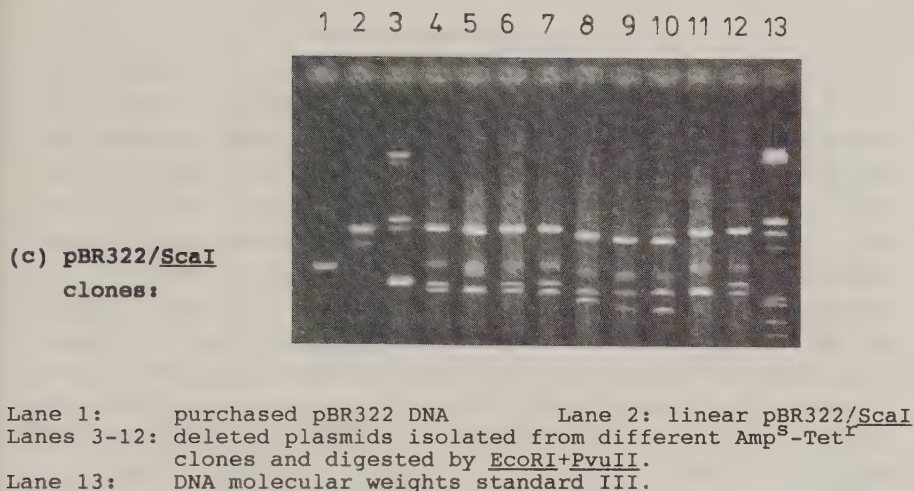


Figure 5 (Contd.): (c) pBR322/ScaI clones (d) pBR322/PstI clones.

N.B. DNA molecular weights standards contains 13 DNA fragments with different Mw which show different resolution mobilities on gel as follows:

Fragment No.	M _w (bp)	Fragment No.	M _w (bp)	Fragment No.	M _w (bp)
1	21,226	6	2,027	10	983
2	5,148	7	1,904	11	831
3	4,973	8	1,584	12	564
4	4,277	9	1,330	13	125
5	3,530				

DISCUSSION

From the results obtained in the present study, the linearization of plasmid DNA at a unique site reduces the transformation efficiency relative to circular molecules from 100 to 1000-fold. The residual minute biological activity was not due to plasmid molecules that had escaped cleavage and remained circular, since the linear form of plasmid was separated and purified before transformation into the bacterial cells. Therefore, the residual biological activity was connected to the re-cyclization probability for some of the linear DNA molecules by the cells. At saturation, the number of linear DNA molecules that could be transformed into cells was relatively higher than that of supercoiled molecules (Fig. 4). This may be due to the permeability difference (of bacterial cells) for each type of DNA or the ability of the cell-enzymes to act on the linear plasmid DNA (repairing or degradation). From the results shown here the dsb produced in the pBR322 plasmid DNA was not a completely lethal damage. The same results were found by several other workers (Thompson and Achtam, 1978; Conley and Saunders, 1984; Bien, 1986 and Conley et al., 1986a). The survival of cells after transformation was considerably higher if the dsb was produced (by restriction enzymes) in the plasmid dimer molecule as found by Symington et al., (1985). The authors showed also that the preferential selection against the establishment of non-recombinant linear plasmids is most likely a result of exonucleolytic degradation of the linear DNA, compared to circular DNA, following transformation. In this respect, one can say that the linear plasmid DNA is degraded in the cell by the action of nucleases but at the same time some of these molecules can be re-cyclized by the repairing enzymes. Krasin and Hutchinson (1981) reported that when repair of dsb was blocked, the genome DNA was degraded by an endonuclease-like action. In addition, evidence was given to show that the inducible inhibition of DNA degradation after X-irradiation (Pollard and Randall, 1973) is probably caused by the inducible repair of DNA dsb. Therefore,

the survival of linear plasmid DNA in E.coli is the result of a balance between degradation and repair, but repair is rare and degradation is the rule (Schulte-Frohlinde, 1987). This balance arises from competition between the action of nucleases and polymerases (Bresler et al., 1984).

Data obtained in Table (3) showed that damage in the Amp. gene gave a reduction in the transformation efficiency similar to that for damage in the tetracycline gene. When different types of linear plasmid DNA were transformed into E.coli, the number of transformants grown on ampicillin plates were nearly similar to that grown on tetracycline plates for each given plasmid (Table 5). Any slight differences in the transformation efficiency between the two antibiotic media were due to the appearance of a few transformants (mutants) which showed sensitivity only to the corresponding antibiotic. The role of the restricted antibiotic gene was limited and only slightly increased the sensitivity to the antibiotic and then the mutation frequency (Table 7). Therefore, the repairing of double strand breaks of the plasmid DNA did not greatly depend on the position of this break. The findings obtained by Symington et al., (1985) showed that altering the position of the dsb in the pBR322 plasmid dimer changed the frequency of recovering different recombination products, but not on the frequency of transformation.

Concerning the type of the restricted ends it was mentioned that the transformation efficiency of some types of linear plasmids depends on the type of restricted ends in these plasmids (Table 3). Linear plasmid DNA with blunt ends gave lower biological activity than that with cohesive ends. The same was found by Bergasma et al., (1982) (BamHI); Thompson and Achtam (1979) (SalI); Conley and Saunders (1984) (SalI) and Bien (1986) (BamHI and PvuII). In the present work, when the cohesive ends of some restricted termini were removed by S1 nuclease to create blunt ends, the transformation of the latter molecules was strongly reduced (Table 4). Similar results were obtained when blunt ends were created in an alternative manner

(by filling-in the single stranded SalI terminal protrusions) with the klenow fragment of DNA polymerase I (Conley and Saunders, 1984). When the restriction enzymes were permeabilized into mammalian cells, the cohesive-ended dsb (BamHI and EcoRI) were less damaging than blunt-ended dsb (PvuII and EcoRV) as reported by Bryant et al., (1987). Similar results were obtained by Winckler et al., (1988) when they used AluI (blunt-ended) in yeast cells.

In the present work the transformation efficiency of some types of linear plasmid DNAs agreed with the previous findings (Table 3). At first, it seemed that the presence of blunt ends was the main factor responsible for the reduction in transformation efficiency of linear plasmids with respect to ccc-plasmids. The limited number of restriction enzyme types (1-3 enzymes) used in the previous studies was not sufficient to test this. We used eight endonucleases which restrict the pBR322 at different positions to give different types of ends. The results obtained in the present study showed an opposite trend to that expected for some types of linear plasmid, since linear plasmid with blunt ends in some cases gave a transformation efficiency similar to or greater than that of plasmids with cohesive ends (Table 3). In addition, linear plasmids with the same type of ends (e.g. cohesive and with the same base extension) gave different biological activity after transformation to cells. Similar observations was reported by Webb and Debenham (1985) and Thacker (1986) who employed the use of cut-plasmid transfer into wild-type and repair-deficient mutant bacteria. They showed that simple restriction cut, whether sticky- or blunt-ended, were found to be joined with equal efficiency and fidelity by ionizing radiation-sensitive mutants (*recN*, *rorA*, *rorB*; the first two of which are known to be strand break repair-deficient) and by their respective wild-type strains. Even when restriction cuts were made with different endonucleases to give non-matching ends in all combinations (e.g. blunt and with 5' sticky end, etc.) these were religated with equal efficiency and fidelity by mutants and wild types. They also added, however, if the cut ends were

treated with phosphatase, so that both kinase and ligase activities must operate to rejoin the breaks, it was found that the *rorB* mutant was two-fold less efficient than the wild type. Thus, it appears that the type of ends is not the only important factor for the repairing of the linear DNA molecules in the cell, but that the base extension and the sequence of the restricted termini may also play a relevant role. This was also apparent from the results of blunt-ends creation (Table 4). Although the base extension which was removed by S1 nuclease was the same in all types of the linear DNA used, the resulting reduction in survival was greatly different for the respective blunt-ended linear plasmids produced. The reduction in biological activity after S1 action is not only due to the creation of blunt-ends but may also be due to the loss of some sequences which are essential for the repairing of the linear DNA molecules. Another reason is that S1 can cleave pBR322 at specific positions which contain inverted repeats as shown by Lilley (1980).

From these results, it was clear that most of the repaired plasmid molecules were perfectly recircularized in the cell and contain both Amp. and Tet. genes. The DNA molecules did not lose the restricted sites and the transformants which contained these plasmids were resistant to both antibiotics. Only a few number of transformants contained plasmid DNA with deletion in the restricted gene, which gave sensitivity to the corresponding antibiotic. This was supported by the results obtained in the experiments that tested antibiotic sensitivity. When the transformants which contained recombinant plasmid DNA were re-examined for antibiotic sensitivity, the sensitivity relative to ccc-plasmid was very low (Table 7). This agreed with the findings obtained by Conley and Saunders (1984); Bien (1986) and Conley et al., (1986a,b).

The linear plasmids which gave lower transformation efficiencies also had higher antibiotic sensitivity and deletion frequencies (Table 7). The data obtained illustrated that the blunt-ends in the linear plasmid or cohesive ends with

short base extension gave more mutation probability than cohesive ends with longer base extension. A portion of transformants obtained with purified linear plasmid DNA contained plasmids bearing deletions that extended various distances from the unique restriction site used for linearization (Table 7). The linear plasmid DNA which produced the highest transformation efficiency (pBR322/PstI) showed the shortest deletion lengths (250-650 bp). On the other hand, the linear plasmid which gave lowest transformation efficiency (pBR322/PvuII) showed the longest deletion lengths (800-2010 bp). We can say that the linear plasmid DNA molecules which give lower transformation efficiency produce the more antibiotic-sensitive transformants (mutants) with longer deletion size and vice versa. This was related to the repairing of the linear plasmid by the intramolecular recombination processes in the bacterial cells (Conley et al., 1986a, see also the restriction and sequencing sections). Bender et al., (1974) proposed that the blunt-ended dsb induced by PvuII (on cell chromosome) are sometimes mispaired, or left unpaired by the cell, leading respectively to exchange and deletion types of chromosomal aberrations. The results of pBR322/SalI were in agreement with the previous phenomenon, since the high deletion percentage obtained was accompanied by a low transformation efficiency. However, the transformation efficiency of this linear plasmid was higher (1.5%) in the work of Conley and Saunders (1984) than that obtained in the present study (0.5%), although the deletion percentage was similar to our value. Thompson and Achtam (1979) observed that the transformation efficiency of Lin/SalI ranged from 0.25-2.5% and the mutation frequency from 20-90%.

The majority of the transformants produced by transforming the linear plasmid DNA to wild type E.coli contained perfectly recircularized plasmid molecules (90-97%) as given in Table (3). The re-cyclization of linear plasmid may performed by ligase action in the cell (Conley and Saunders, 1984). However, the linear DNA molecules with similar restricted ends (same type and base extension) gave different transformation

efficiencies (Table 3). Therefore, the re-cyclization processes depends not only on ligase but also on other proteins in the cell which are essential for the intramolecular recombinational processes. In this respect, we can propose that most of the transformants produced by the transformation of linear pBR322 plasmid DNA to E.coli, arise by intramolecular recombination of head-to-tail linear pBR322 molecules. The deletion in some of the plasmid DNA molecules could be generated therefore during re-cyclization caused by recombination between short homology sequences within a pBR322 molecule (see sequencing section). It is difficult to explain why perfectly recircularized DNA molecules would require recombination functions. Bien et al., (1988) proposed two recombinational pathways for the formation of deleted and non-deleted plasmids (both involving intrachromosomal-site-specific or illegitimate-recombination). When linear DNA recombines, both ends interact with the homologous duplex, resulting in branch migration in opposite directions and the formation of two Holliday junctions (Abastado et al., 1987). Bergasma et al., (1982) showed that a linear plasmid DNA which has one dsb with cohesive terminus in one side and a blunt end in the other side could be repaired. The authors proposed a novel intramolecular end-to-end joining of the linear precursor DNA molecule in which the participants were a blunt end and a 5'cohesive end.

A low transformation efficiency of linear plasmid DNA was found when SFX-recA⁺ and SFX-recA⁻ strains were used as recipient and the data were similar to that of wild-type E.coli CMK (Table 6). These findings agreed with the results of Bien et al., (1988) who reported that the transformation efficiencies showed only a slight dependence on the recA protein. Also Krasin and Hutchinson (1981) observed that the repair of dsb requires more than the presence of the recA protein. In the transformation of linear pBR322 DNA dimer (restricted with BamHI), both the frequency of transformation and the production of (Tet^r) recombinants were decreased by recE mutations, while recA and recF mutations were slightly stimulatory (Symington et al., 1985). However, the results of

Conley and Saunders (1984) showed that the reduction in the transformation efficiency was considerably higher in recA-strains than in the wild type. In this respect, it is possible to suggest that recA may not play a major role in the recombination processes of linear pBR322 DNA. Other factors (in addition to recA) may be essential for the repairing processes of linear plasmid DNA in the cell. The differences in the type of the rec mutant may also play a role. Smith and Sharma (1987) proposed that the resulting dsb (from excised gaps after UV irradiation) becomes susceptible to a recB-dependent repair process. This is analogous to the recB-dependent pathway for the postreplication repair of DNA double-strand breaks that arise at the sites of unrepaired DNA daughter-strand gaps (Wang and Smith, 1983). The recF-dependent and recB-dependent pathways of excision repair are suggested to be analogous to the two major processes of postreplication repair, i.e., in the replicated portion of the chromosome, the RecF pathway repairs gaps, and the RecB pathway repairs the DNA double-breaks that arise at unrepaired gaps.

The repair of linear pBR322 DNA molecules may not depend completely on the genetic background of the cell but on other factors. These factors may include the type of the linear DNA, the type of its ends, the sequence of the end terminus as well as the availability of the enzymes responsible for the repairing processes. Billen (1985) showed that the DNA polymerase I-deficient mutant produced potentially lethal damage when X-irradiated (ssb and dsb present). The authors also observed that DNA ssb and dsb produced by two independent events on opposite strands, as well as base damage were all amenable to repair by the excision-repair pathway involving DNA polymerase I. In this respect, Natarajan and Obe (1984) reported that the inhibition of DNA polymerase α inhibits the excision repair (by inhibition of the ligation step). Their results showed that the addition of the enzyme inhibitor (ara C) with restriction endonucleases increases the frequency of aberrations, indicating that araC affects ligation of DNA double-strand breaks. Bonura et al., (1975) reported that

polymerase I was required for dsb repair (by excision repair). In exonuclease III-deficient (xth^-) E.coli, the transformation frequencies for pBR322/SalI DNA were reduced dramatically in comparison with xth^+ recipients. Also only 5% of the plasmids recovered were perfectly re-cyclized and the remaining 95% had been deleted during the process of recircularization (deleted dimers). However, those juxtapositioned dsb usually produced by a one-track process would require other repair pathways (Billen, 1985). DNA polymerase II and III could be involved in this repair.

CHAPTER 5

**THE PROPERTIES OF MINICLONES PRODUCED IN *E. COLI* AFTER
TRANSFORMATION WITH LINEAR pBR322 PLASMID DNA**

SUMMARY

Supercoiled pBR322 DNA was linearized with some restriction enzymes transformed to *E. coli* and the small size clones (miniclones) which accompany the normal clones were studied. These miniclones began to appear at longer incubation periods than normally required for transformation (16-20 hr) on TBY-Ampicillin plates. They appear in proximity to the normal clones. At longer incubation times, the size and number of the miniclones increased. The miniclones have different sensitivities to the two antibiotics, ampicillin and tetracycline. Three types were detected. The first group was resistant to both antibiotics (Amp^r-Tet^r), the second was sensitive to one antibiotic only (to tetracycline, Amp^r-Tet^s or to ampicillin Amp^s-Tet^r) depending on the restricted gene. While the third group, which represents the majority, was sensitive to both antibiotics (Amp^s-Tet^s). In the latter case most of the miniclones were probably untransformed cells, present due to the large initial amount of cells required for transformation. These grew as satellites of normal clones, due to β -lactamase release from the central colony. This enzyme destroys the ampicillin in the agar media and encourage the satellite formation.

To determine the probability of plasmid loss, the antibiotic sensitivity was tested at different intervals after the appearance of miniclone. Data showed that the growth-efficiency of miniclones (which contain pBR322 plasmid DNA) on the antibiotic media was drastically reduced with time. The mutation frequency was also increased by long incubation and was higher than that in normal clones (10-14 fold). A suggested model for plasmid loss was given (50% loss per division) and supported by the analysis of plasmid DNA in these miniclones. Electrophoretic analysis of the plasmid DNA showed that the

transformation of linear plasmid DNA enhanced the formation of plasmid complexes (dimers, trimers or multimers) which were increased by the plasmid loss.

INTRODUCTION

Double strand breaks in DNA molecules are not easily repaired and lead to cell lethality. When plasmid pBR322 DNA was linearized by gamma rays or restriction enzymes and then transformed to E.coli cells, only a very low number of DNA molecules were recircularized and gave clones on the ampicillin media (Thompson and Achtam, 1979; Garaev et al., 1982; Bien, 1986 and Schulte-Frohlinde, 1987). The authors showed that the repairing efficiency of linearized plasmid was strongly dependent on the structure of the end group (see also Conley and Saunders, 1984 and Conley et al., 1986a). In the present study, the transformation efficiency of linear pBR322 plasmid DNA (produced by different restriction enzymes) was very small. The cells carrying the repaired plasmid showed a heterogeneous clone-size distribution. Small-size clones (miniclones) appeared beside the normal clones.

Cell-size distributions are often observed with mammalian cells. The normal colonies are accompanied by small-size colonies which contain a lower number of cells. In addition, the percentages of these colonies are found to increase by the damage of the cell or its DNA and the colonies produced are very sensitive to the effector (Sinclair, 1964; Nias et al., 1968; Beer, 1979 and Seymour et al., 1986). These studies showed that the reduced size of the colonies produced was due to the slow-growing and poorly plating properties of the cell population. The appearance of small colonies in bacteria and yeast was first suggested by Sinclair (1964).

In bacterial strains harbouring oriC plasmids as deletion derivatives (deleted F'plasmid), many of the plasmids appeared to carry a site of the chromosome called "het". This site leads

to slow growth and heterogeneity of cell size (von Meyenburg et al., 1977; 1978a and 1978b). Ogura and Hiraga (1983) found that by the loss of the mini-F origin which includes ccd (coupled cell division), the cells carrying this deleted plasmid form minute colonies and form fewer colonies than cells lacking the plasmid. To our knowledge no work has been done on bacterial cells in connection with the appearance of small colonies when linear plasmid DNA molecules were transformed to E.coli cells. The appearance of miniclones by linear plasmid transformation encourages us to study the properties of these miniclones. The study was focused on the comparison with the normal clones in connection with antibiotic sensitivity and their contents of plasmid pBR322 DNA.

RESULTS

EFFECT OF RESTRICTED ENDS:

The appearance of small-size clones (miniclones) was seen clearly on plates after the transformation of linear plasmid DNA as compared with that after transformation of the ccc-plasmid (see below). Data in Table (8) shows that the miniclones/normal clones ratio (M/N) was considerably higher for the linear plasmid DNA transformation than for ccc-plasmid transformation. These results also show that the M/N ratio was higher when blunt-ended linear pBR322/PvuII DNA was transformed to CMK cells than when cohesive-ended linear pBR322/BamHI DNA was transformed. However, in the case of linear pBR322/NruI (with blunt ends) the number of normal and mini-clones as well as M/N ratio obtained were nearly similar to that obtained by linear pBR322/BamHI (Table 8). In addition, linear pBR322/PvuI which contains cohesive ends (two base extension) showed an opposite trend and gave data similar to the linear pBR322/PvuII. Therefore, the relative percentages of the miniclones did not generally depend on the type or the position of restricted site in the linear plasmid DNA.

The transformation of ccc-plasmid DNA could also produce miniclones when the plates were incubated for a longer time (≈ 40 hr) or the amount of competent cells (not transformed with plasmid) was highly increased. However, the number of miniclones produced from untreated plasmid and the M/N ratio was lower than that produced from linear plasmid (Table 8). This was found when conditions (e.g. the number of normal clones and of the competent cells) in plates used for the ccc- and linear plasmid DNA were kept equal.

Table 8: Number of normal and miniclones appeared after transformation of *E.coli* with different types of linear plasmid DNA and plating on ampicillin media*.

Plasmid DNA	<u>Restriction</u>		<u>No. of clones</u>		(M/N)** Ratio
	Ends	Gene	Normal	Mini	
pBR322 (ccc)	----	---	4.6×10^5	5.0×10^3 ***	0.01
pBR322/ <u>Bam</u> HI	cohesive	tet	4.4×10^3	2.2×10^3	0.50
pBR322/ <u>Sal</u> I	cohesive	tet	2.4×10^3	1.26×10^3	0.52
pBR322/ <u>Nru</u> I	blunt	tet	3.3×10^3	2.3×10^3	0.69
pBR322/ <u>Tth</u> 1111I	cohesive	---	3.8×10^3	1.5×10^3	0.39
pBR322/ <u>Pvu</u> II	blunt	---	5.0×10^2	7.8×10^2	1.56
pBR322/ <u>Pst</u> I	cohesive	amp	1.4×10^4	7.9×10^3	0.56
pBR322/ <u>Pvu</u> I	cohesive	amp	2.0×10^3	3.36×10^3	1.68
pBR322/ <u>Sca</u> I	blunt	amp	2.3×10^3	1.2×10^3	0.52

* Transformants produced from 1 μ g DNA after 24 hrs incubation and the volume of the competent-cells solution (No. of cells) was equalized.

** M/N = Miniclones/Normal clones ratio.

*** Miniclones appeared after addition of a volume of competent cells equal to that used in plating with linear plasmid (50-250 fold that used with normal plasmid) or the plates were incubated for longer time (see text).

EFFECT OF INCUBATION PERIOD:

To study the effect of incubation time on the number and size of the miniclones, two types of linear plasmid DNA (cohesive-ended pBR322/BamHI and blunt-ended pBR322/PvuII) were used. It was found that a long incubation period of the antibiotic plates after transformation of linear DNA led to an increase in miniclones number while the number of normal clones did not change (Fig. 6a). In addition, the size of these miniclones was increased but still relatively smaller than that of normal clones (Fig. 6b). These changes were more pronounced in the case of linear pBR322/PvuII which gave more miniclones after 24 hr incubation than that of linear pBR322/BamHI.

ANTIBIOTIC SENSITIVITY:

In the experiments, three types of clones were detected, the first was resistant to the two antibiotics ($\text{Amp}^{\text{r}}\text{-Tet}^{\text{r}}$), the second was sensitive to one antibiotic only (to tetracycline, $\text{Amp}^{\text{r}}\text{-Tet}^{\text{s}}$ or to ampicillin, $\text{Amp}^{\text{s}}\text{-Tet}^{\text{r}}$) depending on the corresponding damage to the gene, produced by the specific restriction enzyme. While the third was sensitive to the both antibiotics ($\text{Amp}^{\text{s}}\text{-Tet}^{\text{s}}$).

a) Mutation frequency:

It was found that the growth efficiencies of the re-examined miniclones in an antibiotic (which were taken from ampicillin plates after 24 hr of incubation) were strongly reduced for all types of the linear plasmid DNA tested (82-97%) as in Table (9). This means that most of the cells (of miniclones) may contain deleted plasmid DNA molecules (in the restricted gene) or do not contain any plasmid DNA at all. When the linear plasmid samples were plated onto ampicillin plates, high number of miniclones appeared. These were satellite and scattered colonies throughout the plates. However, the miniclones appearing on tetracycline plates showed lower number (especially with linear plasmid restricted in the ampicillin gene, e.g. pBR322/PstI, pBR322/PvuI and Lin/ScaI) than that appearing on the corresponding ampicillin plates. In this case (i.e. tetracycline plates) only scattered colonies were

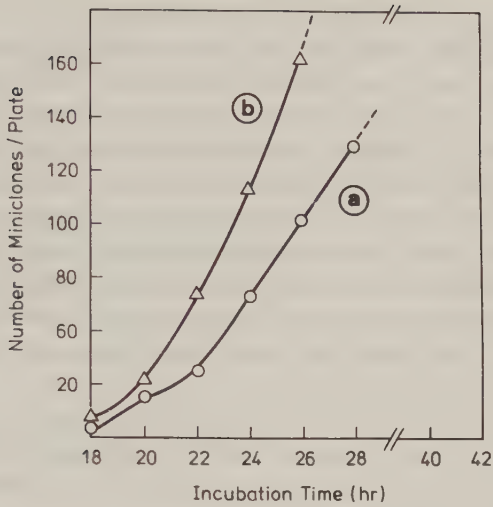


Figure 6a: Number of miniclones produced on transformation of pBR322/BamHI (a) and pBR322/PvuII (b) after different intervals of incubation at 37°C.

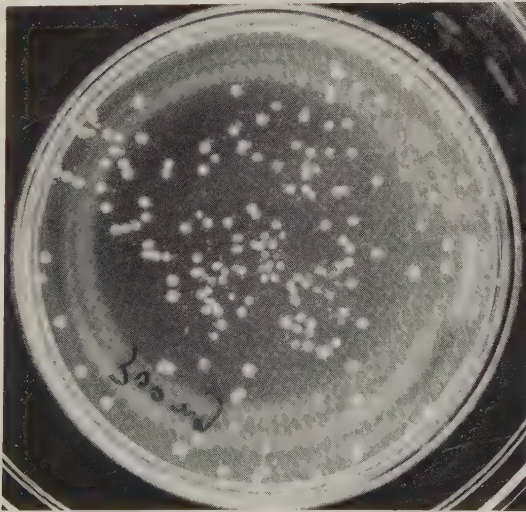


Figure 6b: E.coli CMK clones after transformation of pBR322/BamHI and incubated for 22 hr at 37°C.

observed. These findings indicate that most of the miniclones (77-85%) appearing on the ampicillin plates did not contain plasmid. This may be due to its loss and or due to the miniclones formed from the growth of the non-transformed competent cells. These miniclones grown on the ampicillin plates after the destruction of the antibiotic by β -lactamase. The latter enzyme is formed after induction from the corresponding gene in the normal colonies and leaks into the surrounding. Therefore, the area surrounding the normal colony contains less ampicillin (by the action of β -lactamase) and in turn makes it suitable for growing of the untransformed cells as satellites.

Table 9: Antibiotic sensitivity of the miniclones appearing after 24 hr of transformation of E.coli with different types of linear pBR322 plasmid DNA*.

Original transforming DNA	Total number transformants	Total number sensitive clones	% Sensitivity
<u>a- Transformants taken from amp. plates</u>			<u>Tet^S</u>
Lin/ <u>BamHI</u>	378	329	87.0
Lin/ <u>SalI</u>	384	370	96.0
Lin/ <u>NruI</u>	378	366	97.0
Lin/ <u>Tth111I</u>	384	351	92.0
Lin/ <u>PvuII</u>	378	365	96.5
Lin/ <u>PstI</u>	384	315	82.0
Lin/ <u>PvuI</u>	384	369	96.0
Lin/ <u>ScaI</u>	384	366	95.3
<u>b- Transformants taken from tet. plates</u>			<u>Amp^S</u>
Lin/ <u>PstI</u>	150	5 (1)**	3.3
Lin/ <u>PvuI</u>	150	31 (13)**	20.6
Lin/ <u>ScaI</u>	150	28 (10)**	18.6

* Values were a mean of three experiments.

** Clones showed sensitivity for tetracycline in addition to the ampicillin (Amp^S-Tet^S) due to plasmid DNA loss.

After 24 hr of incubation, the sensitivity for tetracycline gene was high (82-97%) when compared with that for ampicillin gene (3-20%) as in Table (9). If we exclude the miniclones which do not contain plasmid (satellites or by plasmid loss), the deletion frequency for tetracycline gene must be lower than given for the sensitivity in Table (9) and the values may be similar to that for the ampicillin gene. From Table (9) it is also noticed that the miniclones produced from pBR322/PstI had the lowest ampicillin deletion frequency (3.3%). However, this value was 10-times higher than that for the corresponding normal clones (0.3%). In this respect, the deletion frequencies in the miniclones were considerably higher (10-14 fold) than that in the corresponding normal clones (see the restriction of plasmid section).

b) Effect of incubation time (plasmid loss):

Some of miniclones produced on the ampicillin plates (after transformation of linear plasmid DNA) were re-plated onto new ampicillin plates at different times after their appearance. Results showed that the number of the ampicillin-resistant clones decreased with longer incubation periods (Table 10). Therefore, the ampicillin-resistant efficiency was considerably decreased by time, which may be due to the deletion in the ampicillin gene during the repairing processes. However, a deletion in the ampicillin gene could not be obtained in the case of the linear pBR322/BamHI or pBR322/PvuII because the restriction sites are located in/or near to the Tet. gene. Therefore, the increasing ampicillin sensitivity in miniclones containing plasmid restricted in the Tet gene may be due to the loss of plasmid DNA during cell generation. To support this suggestion, the same technique was employed with all types of linear plasmid used. After different incubation periods, each miniclone was re-examined on ampicillin, tetracycline and normal TBV plates at the same time. The data obtained (Table 11) showed that the number of antibiotic-resistant clones was decreased by time. The reduction trend was very similar for

Table 10: Changes in ampicillin sensitivity for miniclones arising after long incubation periods.*

Transforming pBR322/DNA	No. transformants patched	No. of Amp ^r clones appeared		
		18 hr**	20 hr	23 hr
Normal	201 (%)	201 (100)	201 (100)	201 (100)
Lin/ <u>BamHI</u>	201 (%)	49 (24.3)	33 (16.4)	26 (12.9)
Lin/ <u>PvuII</u>	201 (%)	28 (13.9)	22 (10.9)	15 (7.5)
Lin/ <u>PstI</u>	201 (%)	88 (43.7)	62 (30.8)	29 (14.4)

* The miniclones were taken at different times after their appearance and patched onto ampicillin plates. The values were a mean of three experiments.

** This time represents the first appearance of miniclones after 18 hr of transformation of the linear plasmid.

ampicillin and tetracycline which support the suggestion of plasmid loss in the miniclones by cell generation. The reduction in the antibiotic resistance was slightly more pronounced for tetracycline than for ampicillin (specially for the linear plasmid restricted in Tet. gene) as shown in Table (11). However, after longer incubation periods, the reduction in the antibiotic resistance was about the same for the two antibiotics.

The miniclones grown on tetracycline plates after transformation with linear plasmid DNA restricted in the ampicillin gene were re-examined for antibiotic sensitivity. About 30-60% of the miniclones which were tetracycline resistant (Amp^S-Tet^R) showed sensitivity to both antibiotics

(Amp^S-Tet^S) after re-examination (Table 9). These findings support the previous suggestion of the plasmid loss.

EFFECT OF ANTIBIOTIC CONCENTRATION:

The concentration of ampicillin was increased four times in the plate-media before plating cells with transformed plasmid DNA. Data showed that the number of both normal and miniclones was drastically reduced even when the number of competent CMK cells was increased or after longer incubation period (Table 12). It can be suggested that at the higher concentration of ampicillin (>100 µg/ml) the growth-inhibiting effect was stronger (the suggestion may be also right for the induction of β-lactamase).

Table 11: The effect of the incubation time on the antibiotic sensitivity of miniclones produced from transformation of linear pBR322/BamHI*.

Time/hr (37°C)	Total no. of miniclones patched	<u>Amp-sensitive</u>		<u>Tet-sensitive</u>	
		number	(%)	number	(%)
18**	335	260	77.6	288	86.0
19	335	271	80.8	295	88.1
20	335	285	85.0	309	92.2
21	335	290	86.5	326	97.3
22	335	295	88.0	331	98.8
23	335	302	90.1	334	99.7
24	335	327	97.6	335	100
25	335	335	100	335	100

* The number of clones was a mean of three experiments.

** Miniclones start to appear after this transformation time.

Table 12: Effect of ampicillin concentration on the number of grown transformants (normal and miniclones) produced after different periods of transformation of E.coli with normal and linear pBR322/BamHI.

Incubation period/hr	Number of clones* (100 µg/ml Amp.)			Number of clones* (400 µg/ml Amp.)		
	Normal	Mini	M/N**	Normal	Mini	M/N
<u>Normal pBR322 DNA</u>						
18	1.23x10 ⁶	-----	-----	8.2x10 ⁵	-----	---
24	1.27x10 ⁶	2.74x10 ³	0.002	9.13x10 ⁵	1.37x10 ³	0.001
48	1.27x10 ⁶	2.87x10 ⁵	0.220	9.13x10 ⁵	1.64x10 ⁵	0.179
<u>Linear pBR322/<u>Bam</u>HI</u>						
18	2.55x10 ⁴	1.21x10 ⁴	0.47	6.45x10 ³	3.0x10 ³	0.46
24	2.55x10 ⁴	1.50x10 ⁴	0.58	6.45x10 ³	3.3x10 ³	0.51
48	2.55x10 ⁴	6.0x10 ⁴	2.35	6.45x10 ³	1.2x10 ⁴	1.86

* Transformants calculated for 1 µg DNA.

** M/N = Mini/Normal clones ratio.

MICROBIOLOGICAL TESTS:

To study the properties of the miniclones from the microbiological point of view in order to support their strain-characters as E.coli (and not as contamination), some tests were carried out. The tests specific for E.coli strains gave positive results with miniclone cells, while the tests specific for other species gave negative results (Table 13). However, the diagnostic effects in positive tests was relatively weaker for miniclones than that for normal ones. These results indicate that the miniclones are E.coli but may have lower biological reactivity (as mutants).

MINICLONES FROM OTHER STRAINS:

The appearance of miniclones was also detected in E.coli-SFX and SFX recA^- cells when they were transformed by linear plasmid DNA (three different linear pBR322 DNA plasmid were used). Data in Table (14) shows that the miniclones produced after transformation of SFX and SFX recA^- cells gave nearly the same behaviour as CMK cells. In this respect, the F-plasmid did not play a role in the appearance of miniclones.

Table 13: The microbial properties of miniclones compared with those of normal clones*.

Test	Normal clones	Miniclones	Identified microorganism
1) Triple-Sugar Iron Agar Test	++	+	<u>Enterobacteriaceae</u>
2) Christensen-Citrate Agar (modified)	++	+	<u>E.coli</u> + most <u>Salmonella</u>
3) DEV Simmon-Citrate	++	+	<u>E.coli</u>
4) Bactident <u>E.coli</u> Test			
a- β -D-glucuronidase	++	+	<u>E.coli</u>
b- Tryptophanase	++	+	<u>E.coli</u>
5) Vogel-Johnson Test (Vogel-Johnson Agar)	-	-	<u>Staphyococcus aureus</u>
6) Leifson-Agar Test (modified)	-	-	<u>Salmonella</u> , <u>Shigella</u>

* Clones were taken after transformation of E.coli with linear pBR322/BamHI.

The (+) sign represents a positive result for the test and (++) a strong positive result for the test (such as strong colour). The sign (-) indicates negative result for the test.

Table 14: The number of transformants produced after 24 hr of transformation of SFX and SFX $recA^-$ strains with some types of linear pBR322 DNA.

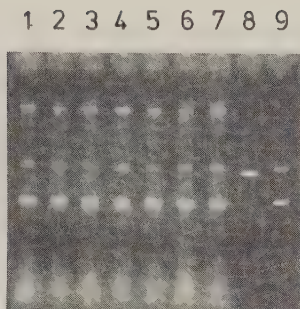
Transforming pBR322 DNA	Number of clones/1 μ g DNA					
	Normal	Mini	M/N	Normal	Mini	M/N
	<u>SFX</u> strain			<u>recA</u> ⁻ strains		
Normal	9.0×10^5	2.8×10^3	0.003	5.5×10^5	6.9×10^3	0.01
Lin/ <u>Bam</u> HI	5.9×10^3	2.8×10^3	0.500	5.7×10^3	3.0×10^3	0.50
Lin/ <u>Pvu</u> II	7.0×10^2	1.8×10^3	2.600	9.3×10^2	2.1×10^3	2.30
Lin/ <u>Pst</u> I	1.6×10^4	3.4×10^2	0.020	1.7×10^4	1.3×10^3	0.07

PLASMID DNA OF MINICLONES:

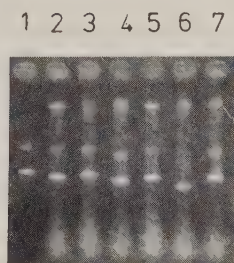
The mini-clones were analyzed for their pBR322 plasmid DNA contents and compared with that of normal clones. The analysis was performed on the clones produced from the transformation of pBR322/BamHI and the results obtained are as follows:

1) The normal clones which were resistant to the both antibiotics (Amp^R - Tet^R) contained normal pBR322 plasmid DNA (Figure 7a). The separated plasmid DNA did not lose the restricted site corresponding to enzyme used for the linearization. The plasmid DNA separated from normal clones which were sensitive only to tetracycline (Amp^R - Tet^S) showed higher mobility (on gel electrophoresis) for both supercoiled and open-circular DNA forms than the ccc-plasmid DNA. These changes were due to the deletion in the Tet. gene of the plasmid (Fig. 7b). In fact, when the separated plasmid DNA was retreated with the enzyme first used for linearization, no linear form was obtained, indicating loss of the restricted site during re-cyclization (repairing) of the linear DNA plasmid in the cell (see the sequencing section). On the other hand, clones which were sensitive to both antibiotics did not contain ccc-plasmid DNA. The supercoiled and open circular forms were not present and at the same time a new band had

(a)
 Lanes 1-7: isolated pBR322 DNA
 from (Amp^{r} - Tet^{r}) clones
 Lane 8: linear pBR322/BamHI
 Lane 9: purchased pBR322 DNA



(b)
 Lane 1: purchased pBR322 DNA
 Lanes 2-7: pBR322 DNA isolated
 from (Amp^{r} - Tet^{s}) clones



(c)
 Lane 1: purchased pBR322 DNA
 Lane 2: linear pBR322/BamHI
 Lanes 3-9: pBR322 DNA isolated from
 (Amp^{s} - Tet^{s}) clones



Figure 7: Agarose gel electrophoresis of pBR322 DNA isolated from normal transformants: (a) Amp^{r} - Tet^{r} , (b) Amp^{r} - Tet^{s} , (c) Amp^{s} - Tet^{s} which are produced after transformation of linear pBR322/BamHI.

appeared on the gel (this band was also present in the other cases but with lower density). This band had lower mobility (on the gel electrophoresis) than that of normal plasmid forms (sc and oc) and was located in the area of multimer plasmid DNA which appeared as traces during normal plasmid DNA separation (Figure 7c).

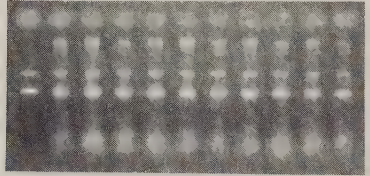
2) The clone analysis for pBR322 plasmid DNA in miniclones gave the same trend as normal clones and the three types of colonies were obtained. The Amp^r-Tet^r type had normal plasmid DNA while the Amp^r-Tet^s had deleted one (Fig. 8a and b). In the third case (Amp^s-Tet^s), no DNA plasmid forms (sc and oc) were detected while the lower mobility DNA band as shown in Figure (8c) was present. It was noticed that the density of the lower mobility DNA band was slightly higher in the case of miniclones than that in normal ones specially in Amp^s-Tet^s class (Fig. 7 and 8).

3) When the lower mobility DNA band was separated and retreated with the same restriction enzyme (BamHI), only slight digestion was detected and most of the band content was not affect by the enzyme. The same was found when the DNA was treated with EcoRI. However, when other enzymes were used (PvuII), most of the DNA contents were digested and it was not possible to detect definite DNA-bands on the gel (as in BamHI, Figure 9a). This may indicate that the restricted site (of BamHI) had disappeared in most of the DNA molecules by a deletion which continued in the anticlockwise direction and had reached the EcoRI position. When the low-mobility DNA band was taken from other miniclones (produced by transformation of linear pBR322/PvuII) a comparable effect was observed. The content of low-mobile DNA was slightly affected (~10%) by PvuII but was completely digested by PstI or ScaI or PstI+ScaI (Fig. 9b).

4) The disappearance of plasmid DNA was noticed also when DNA was separated from most of the miniclones grown on antibiotic plates for long incubation periods (>36 hrs at 37°C, Fig. 10).

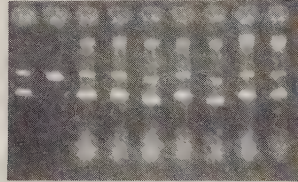
(a)
Lane 1: purchased pBR322 DNA
Lanes 2-11: isolated plasmid from
Amp^r-Tet^r miniclones

1 2 3 4 5 6 7 8 9 10 11



(b)
Lane 1: purchased pBR322 DNA
Lane 2: linear pBR322/BamHI
Lanes 3-9: isolated plasmid from
Amp^r-Tet^s miniclones

1 2 3 4 5 6 7 8 9



(c)
Lane 1: purchased pBR322 DNA
Lanes 2-8: isolated plasmid from
Amp^s-Tet^s miniclones

1 2 3 4 5 6 7 8

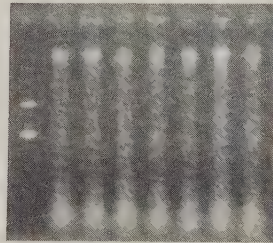
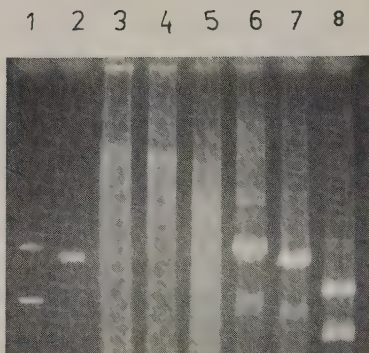


Figure 8: Agarose gel electrophoresis of plasmid DNA isolated from miniclones after transformation of linear pBR322/BamHI.

(a) Amp^r-Tet^r, (b) Amp^r-Tet^s, and (c) Amp^s-Tet^s.

- (a)
- Lane 1: purchased pBR322 DNA
 - Lane 2: linear pBR322/BamHI
 - Lane 3: Amp^S-Tet^S miniclones plasmid after digestion by EcoRI
 - Lane 4: Amp^S-Tet^S miniclones plasmid after digestion by BamHI
 - Lane 5: Amp^S-Tet^S miniclones plasmid after digestion by PvuII
 - Lane 6: Amp^R-Tet^S miniclones plasmid after digestion by EcoRI
 - Lane 7: Amp^R-Tet^S miniclones plasmid after digestion by PvuII
 - Lane 8: Amp^R-Tet^S miniclones plasmid after digestion by EcoRI+PvuII



- (b)
- Lane 1: purchased pBR322 DNA
 - Lane 2: Linear pBR322/PvuII
 - Lane 3: Amp^S-Tet^S miniclones plasmid
 - Lane 4: Amp^S-Tet^S plasmid as in lane 3 after digestion by PstI
 - Lane 5: Amp^S-Tet^S plasmid as in lane 3 after digestion by PvuII
 - Lane 6: Amp^S-Tet^S plasmid as in lane 3 after digestion by PstI+ScaI
 - Lane 7: Standard DNA marker II

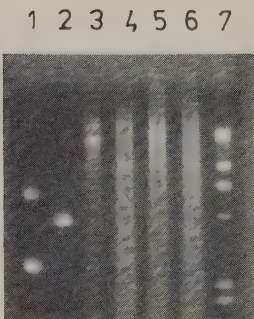


Figure 9: Agarose gel electrophoresis of plasmid DNA isolated from miniclones and treated with different restriction endonucleases.

(a) miniclones produced by transformation of linear pBR322/BamHI,
 (b) miniclones produced by transformation of linear pBR322/PvuII.

In the samples tested, only the lower mobility DNA band was detected.

5) When SFX or SFX-recA⁻ strains were used for transformation of linear pBR322 plasmid DNA, the clone analysis results obtained (for plasmid DNA) were similar to that obtained from CMK strains.

6) The cells of the three strains, CMK, SFX and SFX recA⁻ (not transformed with plasmid) were analyzed to compare the results with that of the same cells transformed with linear plasmid DNA. The results obtained showed that the cells of these strains did not contain normal pBR322 DNA plasmid but contained traces of a low-mobility band near (but not exactly) to the area of lower mobility DNA band observed in transformed clones (Figure 11).

DISCUSSION

The presence of the miniclones requires explanations as to the reason for their appearance, the factors affecting their growth and properties as well as their content of the plasmid DNA. As previously mentioned from literature, the appearance of small-size colonies was observed in mammalian cells and their abundance relative to normal colonies was increased by treatment of the cells or by affecting their DNA. These studies showed that the appearance of these small-size colonies was due to a decreased number of viable cells in the colony as compared to that in normal-size colonies. This resulted in a slow growing rate (less dividing efficiency) as reported by Sinclair (1964) and Beer (1979). The studies of Todd (1968) also showed that the number of cells in the small-size colonies depends on the type of effect or damage as well as the type of cells. The properties of the small colonies in mammalian cells are different than that of normal colonies since they are very sensitive to retreatment with the effector first used on the cells. These mammalian colonies showed also some morphological changes which could be related to the occurrence of heritable

Lane 1: purchased pBR322 DNA
Lane 2: linear pBR322/BamHI
Lane 3: Amp^r-Tet^r miniclones plasmid
Lane 4: Amp^r-Tet^s miniclones plasmid
Lane 5: Amp^s-Tet^s miniclones plasmid

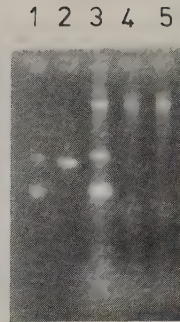


Figure 10: Agarose gel electrophoresis of plasmid DNA isolated from miniclones produced by linear pBR322/BamHI transformations and incubated for 40 hr.

Lane 1: purchased pBR322 DNA
Lane 2: linear pBR322/BamHI
Lane 3: DNA isolated from CMK
Lane 4: DNA isolated from SFX
Lane 5: DNA isolated from SFX recA⁻

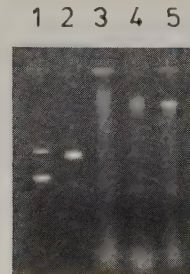


Figure 11: DNA isolated from different types of E.coli strains (not transformed with plasmid).

lesions as well as some chromosomal aberrations (Stroud and Resh, 1967).

In the present study, some of the miniclones (at least 20%) appeared only after transformation of linear plasmid DNA but not with ccc-plasmid DNA. These findings lead us to believe that the damaging effect produced by enzymes (dsb) may be similar in effect to that produced in the DNA of the mammalian cells by irradiation or chemical treatments. In addition, the sensitivity of the miniclones to the antibiotic (ampicillin or tetracycline) may be comparable to the sensitivity of small-size mammalian-cell colonies to the effector used on the cell. The data obtained here showed that some of the miniclones contained deleted plasmid molecules (aberrant) causing sensitivity to the antibiotic corresponding to the gene. This leads us to suggest that the damage or loss of plasmid DNA (partly or completely) in these cells was responsible for the appearance of miniclones.

From the results obtained here, we suggest the probable appearance of the three types of miniclone as follows:

A) AMP^r-TET^r MINICLONES:

These are clones which contain perfectly recircularized pBR322 plasmid DNA and represent about 14-18% of the total miniclones in the earlier stages of their appearance. The small size of the clones may be due to the slow-growing rate of the cells in comparison with the normal clones. This slow-growing rate may be related to one of the following reasons: a) The slow rate or yield of recircularization of the linear DNA plasmid (low repairing efficiency). b) A low copy number of plasmid DNA molecules which may be due to the loss of most of these molecules during the incubation period. c) The clones may have a certain sensitivity to the antibiotic that could not be detected in the present study (the number of generations was relatively low). This sensitivity does not appear directly (may be due to a very short deletion which could not be detected by gel electrophoresis) but may appear after many generations of

the cells. This sensitivity may affect the cells and give lethal heritable damage which appears with time. This phenomenon was first observed by Seymour et al., (1986) for the miniclones in mammalian cells.

B) AMP^r-TET^s- OR AMP^s-TET^r MINICLONES:

This type represents the lowest percentage (3-6%) of the total miniclones. In these clones, most of the pBR322 plasmid DNA molecules showed a deletion in the restricted gene and the restricted site had been lost as shown in Fig. 8b and 9b (lanes 6-8). These deletions increased the sensitivity to the corresponding antibiotic which may have reduced the growth rate of the clones, the copy number of the plasmid DNA and or enhance the plasmid loss. These effects may in turn slow the growth rate of the cells on the antibiotic media and produce small-size clones (miniclones).

The work of Zyskind et al., (1979) showed that the deletion derivatives of the plasmid carrying the replication origin of S.typhimurium chromosome could be lost during cell growth. The rate of loss was inversely correlated with the copy number of the plasmid. The authors added that cultures of cells containing plasmids that were lost at high rates exhibited abnormally longer generation times and contained many antibiotic-sensitive cells. In addition, von Meyenburg et al., (1978) found that transductants originating from infection with asn (gene from phage) not only had a low level of *oriC* plasmid DNA and were unstable but also showed slow growth and heterogeneity of cell sizes, i.e. disturbed cell division. They suggested that the site corresponding to *het* locus had a major regulatory function in chromosomal DNA replication. Ogura et al., (1980) found that the plasmids carrying the replication origin (*oriC*) of the E.coli chromosome could be lost in some of the cells during 10 generations when these had a specific region in the *oriC* segment. This region, designated *cop* (copy number) acted to reduce the copy number of the plasmid, since the *cop*⁻ mutants were found to carry a higher copy number of plasmid. In the present study, the microbiological tests showed

that the miniclones have lower metabolic efficiencies than normal E.coli cells. These findings may be due to the long deletion in pBR322 plasmid or to the lower copy number of the plasmid in these miniclones.

c) AMP^S-TET^S MINICLONES:

This class of clones represents the majority (76-83%) of the total miniclones that appeared and they do not contain normal pBR322 plasmid DNA. The disappearance of the plasmid DNA in the clones may be due to its loss and or to the fact that the cells did not contain the plasmid in the first place (non-transformed competent cells). The latter is probably responsible for the appearance of the satellites which surround the normal clones. These satellites were found to represents 87-91% of the miniclones which were sensitive to both antibiotics (Amp^S-Tet^S). The mechanism resulting in these miniclone types is as follows: In the transformation of linear plasmid DNA technique, a high concentration of competent CMK cells must be used in plating (because of the transformation efficiency of the linear plasmid). This high volume of the competent cells contains a higher number of cells (50-250 fold) to that used in the plating of normal plasmid DNA. The high number of the non-transformed competent cells increases the probability of the formation of CMK satellites. Therefore, these miniclones were normal E.coli CMK which were not transformed by plasmid pBR322 DNA and their growth was allowed after ampicillin destruction in the plates as an artefact of the high CMK concentration. The antibiotic could be destroyed by long incubation times at 37°C or by β -lactamase secretion from normal transformants. Maniatis et al., (1982) recommends that in the transformation experiments the density of cells plated should be low and the plates should be removed from the incubator after 16-24 hours. β -Lactamase secreted into the medium from ampicillin-resistant transformants, rapidly depletes the antibiotic in regions surrounding the colonies. Thus, plating cells at high density or incubation for longer periods would lead to the appearance of ampicillin-sensitive satellite colonies. This suggestion is in agreement with

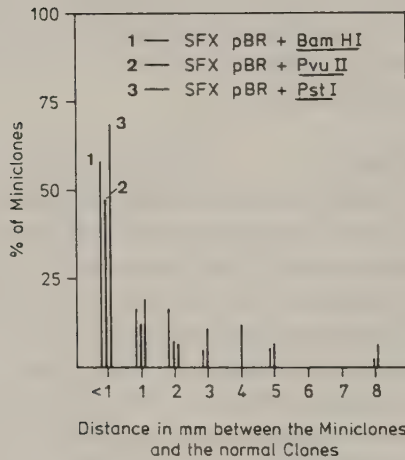


Figure 12: The relative distribution of miniclones as a function of their distance from normal clones (Steffen, H. personal communication).

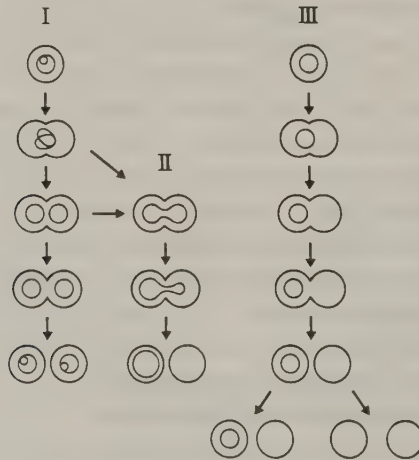


Figure 13: Model for plasmid loss. The model postulates 3 pathways for plasmid partitioning: I- No plasmid loss. II- In the absence of specific recombination functions (as presented by Austin *et al.*, 1981). III- 50% plasmid loss (per generation).

Maniatis et al., (1982) and with data in Figure (12). In support of these proposals the incubation of plates containing ccc-plasmid transformants, when extended to 40 hrs also resulted in the appearance of satellites. Furthermore, increasing the concentration of competent cells was also found to result in miniclones appearance with ccc-plasmid transformants in some experiments. The appearance of miniclones after transformation of the normal plasmid in response to high competent cell-concentration varied considerably from one experiment to another. However, when the normal clones were re-examined on new antibiotic plates previously plated with high competent cells, satellite appeared. The suggestion that the appearance of miniclones is an artifact of high CMK concentrations can not therefore be ruled out at least for the miniclones which are very close to and surround the normal clones. A longer incubation time will increase the number of this type of miniclones (satellite) due to the increased β -lactamase release.

As the sensitivity to tetracycline and ampicillin also increased with time it appears that some of the Amp^S-Tet^S miniclones (at least 10%) were due to cells which had lost their plasmid DNA (Figure 13). The loss could happen during the expression time or during the re-examining process for antibiotic sensitivity (see Table 9). The loss of plasmid in these miniclones may be connected with the increase of the multimer forms which was noticed on electrophoretic analysis (the low mobility band, Figure 7 and 8).

Krivosnogov (1984) reported that the stability and monomerization of supercoiled pBR322 DNA were controlled by recF⁻ dependent endonuclease from E.coli K12. The results demonstrated that an endonuclease activity of the recF⁻ controlled protein Z, might be involved in general recA⁻ dependent recombination and formation of the pBR322 monomers in the cells. The authors found that strains deficient in recF endonuclease, such as recBC sbc recF 144 cells, contained plasmid multimers (circular oligomers) in about 60% of

transformants immediately after transformation. The other clones contained a portion of monomeric molecules which disappeared after 50-70 generations of cell growth. In other mutant strains (rec 143 cells), plasmid DNA molecules were presented by dimers, trimers in addition to the multimers. The results of Krivonogov (1984) supported the correlation between the level of plasmid elimination and plasmid multimerization which was initially observed by Hakkaart (1982). Conley et al., (1986a) showed that when E.coli was transformed with linear plasmid pBR322 DNA molecules, some transformants contained aberrant plasmids which were larger than the monomer plasmid (>monomeric but <dimeric and >dimeric but <trimeric). The inverted dimeric and trimeric plasmids were also detected. The content of these plasmid-classes (with deletions) were increased in recombination-deficient strains of E.coli. The work of Cassuto et al., (1980) showed that the pairing of duplex DNA molecules (in different types of DNAs including pBR322) could be promoted (by recA protein) when some of these molecules contain one or several small single-stranded regions. The authors observed that, with DNA preparations containing a single gap per molecule, the product of the reaction is a dimeric structure, and its efficiency does not seem to be affected by the size of the gap, at least for gap lengths between 65 and 750 nucleotides. While with DNA preparations containing several gaps per molecule, products containing a large number of plasmid units are formed. Similar findings were recently reported by Herskind (1987) who observed that single-strand breaks can lead to complex configurations of plasmid DNA in vitro. In 1987, Shishido et al., found that pBR322 DNA plasmid molecules were produced as knotted species in the E.coli topoisomerase mutants (i.e. closely related to mutations of the gyrase genes). Also, the formation of biparental figure-eight molecules from plasmid DNA and their resolution in E.coli is probably possible as reported by West et al., (1980). In this respect the miniclones produced after transformation with linear DNA plasmid in the present study may be mutants and contain plasmid complexes similar to one or more of those mentioned by the above authors. In addition, the formation of

anomalous plasmid types may explain the difficulty we faced in characterizing the low mobility band.

In conclusion we have observed the appearance of miniclones of three types. To explain this we propose that the miniclones consist either of transformants with some form of plasmid damage (17-24%) and the majority due to cells without plasmid, which were allowed to grow due to β -lactamase release by normal clones. The latter consist mostly of CMK competent cells (90%) but in part due to transformants that have lost their plasmid (10%). It is interesting to suggest a mechanism for plasmid loss during cell generation by a 50% loss model (Fig. 13). In the absence of specific recombination functions, multimeric plasmid molecules were accumulated resulting in the generation of plasmidless cells (Austin et al., 1981). In the case of transformed cells the loss of one antibiotic gene (partly or completely by deletion) leads to the late repair of the linear plasmid in some cells. This will produce a low cell-growth on the antibiotic medium and in turn gives small-size colonies (miniclones). The deletion in the plasmid molecules enhances its instability in cells which then lose their antibiotic resistance with time. The deleted plasmid could disappear immediately after the transformation or after a longer time of incubation and depends on the repair mechanism for the plasmid in the cell. The low metabolic efficiency and the high antibiotic sensitivity of the miniclones may be due to the low copy number of plasmid DNA in these cells. The destruction of the antibiotic gene (deletion) in the plasmid DNA followed by its loss, may be accompanied by the formation of anomalous plasmid molecules.

A more complete mechanism for the appearance of miniclones, especially in connection with chromosomal DNA and the characterization of plasmid polymers formed on plasmid loss require more work.

CHAPTER 6

NUCLEOTIDE SEQUENCE OF THE DELETION END-POINTS OF PLASMID
DELETANTS FORMED BY INTRAMOLECULAR RECOMBINATION FOLLOWING
TRANSFORMATION OF ESCHERICHIA COLI WITH LINEARIZED DNA

SUMMARY

The transformation of E.coli K-12 CMK strain by various linear forms of pBR322 DNA produced many transformants which contained recircularized plasmid bearing deletions. The deletion end-points of three plasmid deletants isolated from tetracycline sensitive transformants (from pBR322/BamHI) were determined by DNA sequencing. The sequencing data showed that only one deletant had bidirectional deletion while the others had unidirectional deletions. In the bidirectional deletant 582 bp of DNA were lost while, 1327 and 1656 bp were lost in the two unidirectional deletants. The restriction site for linearization (BamHI) was lost completely in the bidirectional deletant, and partly in the unidirectional deletants.

The end-points of the deletion in the two unidirectional deleted plasmids occurred at positions 375 and 1707 bp & 375 and 2036 bp from the EcoRI site respectively. The end-points of the deletion in the bidirectional deletant occurred at positions 359 and 942 bp from the EcoRI site. For the repair of the linearized plasmid DNA which leads to deletion, a homologous sequence is required. The recyclization point in both unidirectional and bidirectional deletions was characterized by recombination between directly repeated sequences of at least five uninterrupted bp on one or both arms of the linearized pBR322. The computer analysis of the homology sequences (coincides with the sequence-termini) showed some discriminations for the possibility of different deletants production. The ability of deletion formation correlates with the extent of homology between the short repeated sequences although other factors may be involved. The direction of

deletion, clockwise and or anticlockwise depends strongly on the direction of the terminus which is responsible for the matching homology formation.

INTRODUCTION

The transformation efficiency in E.coli by linearization of the plasmid pBR322 DNA molecules was drastically reduced (Thompson and Achtam 1979; Conley and Saunders 1984 and Schulte-Frohlinde 1987). From the transformants produced a varying proportion contained perfectly recircularized plasmids (about 95%). However, some transformants contained plasmids that bear deletions or other sequence rearrangements (Garaev et al. 1982; Conley and Saunders 1984; and Bien 1986). Conley et al., (1986a) used linear plasmid DNA as a model substrate for analysing the fate of linear recombinant plasmid molecules that would enter E.coli following transformation with a ligation mixture. The authors showed that in addition to perfectly recyclized plasmids, three classes of aberrant plasmids were detected in various recombination-proficient and deficient strains of E.coli.

For the repair of the enzymatically linearized DNA two recombinational pathways were reported by Conley et al., (1986a) and Bien et al., (1988). The first pathway leads to deletant transformants while the second leads to non-deletant transformants. The type of recombinational pathway and the length of a deletion depends strongly on the sequence of the restricted site in the linear plasmid DNA. In the present work we have used pBR322 DNA linearized with BamHI as a substrate for the generation of various types of deletants. We describe here the sequence analysis of the deletion termini of three deletants, and propose a model to explain the deletion process during recircularization of the linear plasmid molcules in E.coli.

RESULTS

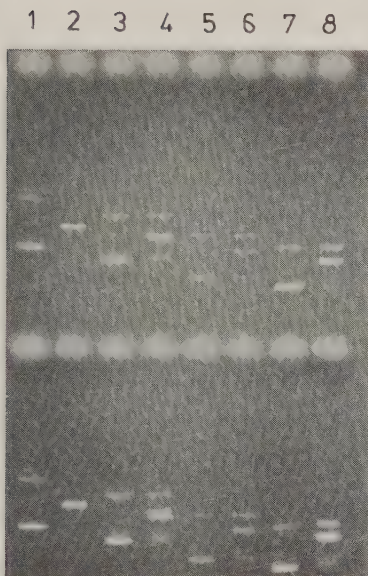
The plasmid vector pBR322 has 4363 bp and confers resistance to ampicillin and tetracycline on the host bacteria (Bolivar et al., 1977). The unique restriction site of BamHI is located in the Tet. operon; therefore, the clones carrying plasmid with the BamHI deletion selected among the Amp^r-Tet^s transformants.

The analysis of Amp^r-Tet^s clones revealed a large group of transformants containing plasmids of relative molecular weight values (M_r) smaller than that of the vector plasmid. The different appearance of Amp^r-Tet^s clones containing this type of deletion are shown in Figure (7b). Three different Amp^r-Tet^s clones were selected for deletion analysis using the sequencing technique in comparison with the normal plasmid.

EcoRI, EcoRV, BamHI, SalI, NruI and AvaI were used for both normal and the three tested deletants. The treated plasmids were electrophoresed on agarose gel and the results obtained are given in Figure (14,a-f). The three deletants were not affected by SalI which restricts plasmid at position 651 (about 278 bp distant clockwise from BamHI site) which means that the deletion in all the deletants tested is greater than 300 bp in the clockwise direction. When NruI was used only the first deletion (D1) type gave a linear form which means that the deletion in D1 is ended before the NruI site (974) while the other two deletants are extended further than the 974 site. The same trend was found by using AvaI, indicating that the deletions in the D2 and D3 are extended further than the 1425 site. On the other hand, the three deletants are not restricted by BamHI which means that the restricted site of the enzyme was lost. But it is not clear whether the loss was complete or incomplete which is important for distinguishing the type of deletion (unidirectional-or bidirectional). To solve this problem the sequencing of the joining points for each deletion was performed.

(a) EcoRI

(b) EcoRV



Lane 1: pBR322 DNA

Lane 2: pBR322 DNA treated
with the enzyme

Lane 3: D1 plasmid

Lane 4: D1 plasmid treated
with the enzyme

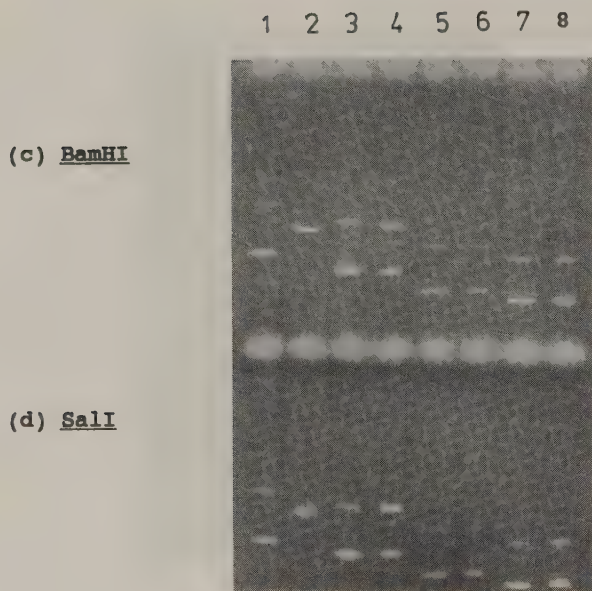
Lane 5: D2 plasmid

Lane 6: D2 plasmid treated
with the enzyme

Lane 7: D3 plasmid

Lane 8: D3 plasmid treated
with the enzyme

Figure 14: Agarose gel electrophoresis of the three isolated deletants D1, D2 and D3 after treatment with some restriction endonucleases: (a) EcoRI, (b) EcoRV, (c) BamHI, (d) SalI, (e) NruI and (f) AvaI.



Lane 1: pBR322 DNA
Lane 2: pBR322 DNA treated
with the enzyme
Lane 3: D1 plasmid
Lane 4: D1 plasmid treated
with the enzyme

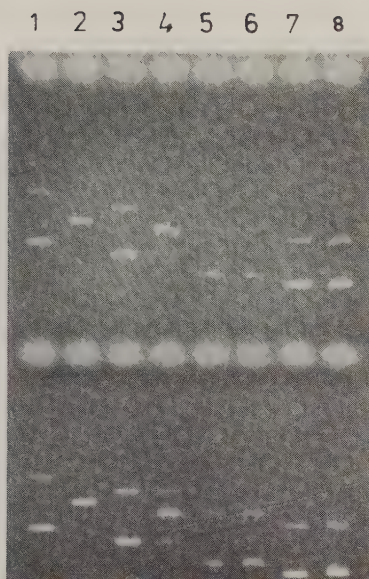
Lane 5: D2 plasmid
Lane 6: D2 plasmid treated
with the enzyme
Lane 7: D3 plasmid
Lane 8: D3 plasmid treated
with the enzyme

Figure 14: Continued. (c) BamHI

(d) SalI

(e) NruI

(f) AvaI



Lane 1: pBR322 DNA
Lane 2: pBR322 DNA treated
with the enzyme
Lane 3: D1 plasmid
Lane 4: D1 plasmid treated
with the enzyme

Lane 5: D2 plasmid
Lane 6: D2 plasmid treated
with the enzyme
Lane 7: D3 plasmid
Lane 8: D3 plasmid treated
with the enzyme

Figure 14: Continued. (e) NruI

(f) AvaI



Figure 15: Autoradiograph of dATP ($\alpha^{32}\text{S}$) reactions on a THE gradient gel for D1-deletant plasmid. The lane order from left to right is T C G A. The three sequences are presented for three different primer concentrations (from left to right: 0.1, 0.5 and 1 pM respectively).

SEQUENCE DETERMINATION OF D1 END-POINTS:

The autoradiography of the sequencing gel of the deletant D1 is given in Figure (15). The figure was given as an example, and to show the effect of primer concentration. This was essential in order to get a clear autoradiograph and in turn accurate readings of the sequence ladders. We used the gradient gel system of Biggin (1982). From this figure 200-250 nucleotides have been read around the endpoints of the deleted plasmid.

The endpoints sequence of D1 is given in Figure (16). The deletant consists of the monomeric pBR322 sequence with a 582 bp bidirectional deletion extending from base 360 to 941 (from the EcoRI site). From the sequence obtained, it was noticed that the sequence of BamHI termini (375-380) had disappeared. It is not easy to distinguish the position of the sequence -CC- in the junction site, therefore, it is better to say that the deletion extends from 360-362 bp to 941-943 bp.

359

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5'-CTACGCGATCATGGCGACCCACACCCGTCCTG-3'
3'-GATCGGCTAGTACCGCTGGTGTGGGCAGGAC-5'
      ***      *
5'-TATCGCCGGCATGGCGGCCGACGCGCTGGGCT-3'
3'-ATAGCGGCCGTACCGCCGGCTGCGCGACCCGA-5'
      942
  
```

Figure 16: Deletion end-points of D1 deletant (bidirectional). Sequences not underlined are deleted as a consequence of the re-cyclization process. Asterisks represent points of sequence match.

The figure illustrates that the deletion end-point is localized at regions in either arm that are much more homologous to each other (9 out of 10 bases match in the regions spanned by base positions 352 to 361 and 934 to 943). This region of joining (352 to 943) showed 90% homology and this percentage is decreased by increasing the length of paired strands as follows: 75% and 58% for 16 bp and 24 bp lengths respectively.

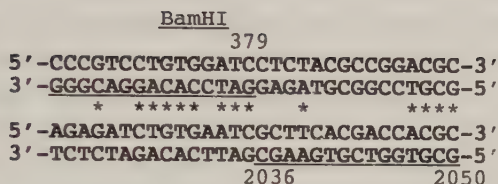


Figure 18: Deletion end-points of D3 deletant (monodirectional). Sequences not underlined are deleted during re-cyclization process. Asterisks represent points of sequence match.

At the end point of the deletion there is a match homology between the paired DNA strands by an interrupted 8 base pairs (5+3 interrupted by one base pair) in one side. The other side has also a small homology (1+4) which may be useful for the fixing and stabilizing the four DNA strands at the joining region for the recombinational repair of the linear plasmid. The percentages of homology at the joining site are 89% for 9 bp length at the critical area (371 to 379) and 62.5% for 16 bp length (368 to 2039) while for longer length at both sides (27 bp) the percentage is reduced to 52% homology. The three tested deletents have differnt mobilities on agarose gel electrophoresis which represent different relative molecular weights (M_r) due to different deletions as a sequence of the recyclization processes (see Fig. 7b). The results obtained for these deletants are summerized in Table (15).

DISCUSSION

In the previous sections, when linear plasmid pBR322 DNA molecules was transformed to E.coli, the effeciency of transformation was very low. This was also found by Thompson and Achtam 1979; Conley and saunders (1984), Conley et al., (1986a) and Bien et al., (1988) and they added that the proportion of deleted to perfectly re-cyclized plasmids was low. This means that only a small proportion of linear DNA

molecules survives processing by the enzymatic machinery involved in re-cyclization and deletion. This might explain both the higher molar demand for linear as opposed to circular plasmid DNA and the relatively high frequency with which aberrant plasmids are formed in transformants. The low survival of linear plasmid is due to its fast degradation with nucleases (as exonuclease VIII, reported by Symington *et al.*, 1985). The degradation of linear plasmid is more frequent than its

Table 15. Properties of the plasmid deletants obtained from *E.coli* after transformation with linear pBR322/BamHI.

DELETANT/ PROPERTIES	D1	D2	D3	NORMAL PLASMID
ANTIBIOTIC EFFECT	Amp ^r -Tet ^S	Amp ^r -Tet ^S	Amp ^r -Tet ^S	Amp ^r -Tet ^r
MOLECULE: bp,	3781	3036	2707	4363
M _r ,	2.42x10 ⁶	1.95x10 ⁶	1.74x10 ⁶	2.8x10 ⁶
DELETION: type,	bidirect.	unidirect.	unidirect.	----
direction	(----	(clockwise)	(clockwise)	
length, bp,	582	1327	1656	----
extension,	(360-940)	(380-1706)	(380-2035)	----
HOMOLOGY:				
uninterrupted,	7 bp	8 bp	5 bp	----
interrupted,	9 out of 10 (90%)	13 out of 16 (81%)	8 out of 9 (89%)	
<u>BamHI</u> SITE:	100% loss	17% loss	17% loss	no
loss				
sequence,	----	GGATC	GGATC	GGATCC
enzyme effect,	-	-	-	+

repairing (Seroke and Wackernagel, 1977). We can also assume that the survival of linearized plasmid might need a complicated system for repairing including different enzymes and proteins.

For the perfect re-cyclization of the linearized plasmid DNA molecules the repair might occur by circularization either spontaneously or enzyme-induced inside the cell and then by rejoining through simple ligation. Conley and Saunders (1984) observed that the transformation efficiency was three times higher in an E.coli strain overproducing its ligase in comparison to the wild type strain. On the other hand, the formation of deleted plasmids demonstrates that in addition to the simple ligation, there occur repair by site-specific (Garaev et al., 1982) or illegitimate recombination (Low and Porter 1978). The repair of linear plasmid proceeds through the intramolecular repair via two major recombinational pathways, one for the formation of perfectly re-cyclized plasmids and the second for the deleted plasmids (Bien et al., 1988). The linear plasmid recombination in wild-type E.coli occurs via a pathway similar to the RecF pathway (Symington et al., 1985), since this pathway responds to double-strand cuts (Abastado et al., 1987).

The sequence of the termini is essential for promoting pairing interactions between homologous DNA strands which stimulates the intramolecular recombination and in turn production of the deletions. The three deletants studied have different deletion lengths, D1 is short (582 bp), bidirectional and most of the deletion sequence is clockwise. The D2 and D3 are longer (1327 and 1656 bp respectively), both are unidirectional and the deletion sequences are clockwise. We do not know the reason for these differences and many questions are raised: How long must the homology sequence be for both uni- and bidirectional deletions; Why are the detected DNA-deletants clockwise, although Conley et al., (1986b) have found the opposite trend by using linear pBR322/SalI plasmid?. What

are the factors affecting the length of deletions in the same direction (unidirectional) which contain the same sequence at one end point? Why does the BamHI not restrict the D2 and D3 plasmid although most of the termini sequence exists?.

To answer these questions, the re-cyclization of linearized plasmid molecules was dependent on the nature of the DNA termini involved, as found in the restriction enzymes-section (see also Schulte-Frohlinde, 1987). The recircularization efficiency of linear pBR322/BamHI (cohesive-ended) was about 10 times higher than that of linear pBR322/PvuII (blunt-ended). This may be due to the higher ligation probability of the cohesive ends than that of the blunt ends. However, this is not invariably the case, since our previous results showed that some blunt-ended linearized plasmid had higher (or similar) transformation efficiency than some others with cohesive ended-termini. In this respect, not only the type of ends is important for repairing of linear plasmid but also the sequence of these ends. Conley *et al.*, (1986a) reported that there were apparently many more potential sites for recombinational re-cyclization and deletion in linear pBR322/SalI molecules bearing cohesive termini than in blunt-ended linear pBR322/PvuII molecules. They showed that this might reflect a higher proportion of short homology matching events with SalI termini. To apply these observations with linear pBR322/BamHI and linear pBR322/PvuII, the interpretation is difficult to understand. The observed computer analysis showed that the number of regions which contain matching homology sequence with the BamHI termini were lower (about 14 regions) than that found for PvuII termini (about 45). The computer screening extended from positions 1-2500. The high number of homology sequences found in the case of PvuII may explain the high deletion frequency (about 90%) which was obtained by Conley *et al.*, (1986a) and Bien *et al.*, (1988). Our data are consistent with the results of Abastado *et al.*, (1987) who that showed double-strand breaks stimulate recombination, and they do so only when the break lies in a region of homology. They added that a recombination signal propagates from the break and that the

introduction of a piece of heterologous DNA in the homology region creates a hot spot of recombination nearby.

The results presented here illustrated that the end points of the three deletants were determined by matching of short DNA sequences that happened to occur fortuitously in both arms of a transforming linear plasmid molecule (Figures 16,17 and 18). The sequence obtained for D2 and D3 revealed that the deletion started after 379 bp although the BamHI restricts the plasmid DNA molecules at 375 bp site. This can be described as base filling for the short strand (5'→3') of the BamHI-cohesive termini after deletion and the ligation of the daughter strand of the termini with the other end point during the recombination processes (Figure 19a and b). From this proposed reaction mechanism it can be understood that although the BamHI cuts at 375 bp site, most of the sequence of the termini (375-379) was detected in the sequence of the deleted plasmid obtained (D2). Also one can recognize why BamHI is not able to restrict this DNA. The site sequence of the enzyme in the deletant is not complete (base no. 380 was lost). The deletion was extended from the other end of BamHI (clockwise) until the recognized homology region (1697-1713 pb). The primary sequence of the donor strand defines appropriate short homologies in the remainder of the molecule with which it has the opportunity to recombine. The base filling of the gap-trend was also expected for the D3 deletant (the same 5 base termini of BamHI were detected). A similar mechanism for the gap filling has been given by Bergsma, *et al.*, (1982). They observed the ability of ligation and base filling between two dissimilar termini of linear DNA, one containing the blunt-ended/PvuII terminus and the other containing the cohesive-ended/BamHI terminus.

Garaev *et al.*, (1982) separated one deletion derivative as a result of transformation of E.coli cells by the linear plasmid (pBR322/BamHI). The endpoints of this deletion occurred at positions 375 and 1666 bp from the EcoRI site. This deletion derivative is very similar to the deletion D2 obtained in this study, but its deletion length is slightly shorter (~ 36 bp).



Figure 19a: A proposed mechanism of D2 formation involving space filling of BamHI sequence.



Figure 19(b): Proposed mechanism of D2 formation as an example for the unidirectional deletions. The scheme is based on the work of Conley *et al.*, (1986b)

The deletant had lost the restriction activity for BamHI although only one base was lost (380) from the sequence terminus. This is in good agreement with the results obtained for this type of deletion (D2 and D3). The authors showed that the formation of the deletant was most probably due to site-specific recombination between the sequence in the 1666-1670 bp region and the BamHI end of the linear pBR322 molecule. In this region a matching homology sequence of 5 bp was detected and it was similar to the BamHI sequence region (Table 16).

The different deletion lengths in deletants of the same type (same direction and starting deletion point) may depend upon the relative percentages of homology needed as well as on the competition between the enzymes during recombination of the linear pBR322 DNA. From the BamHI site (375-380 bp) until the 2040 bp site, there are nine sequence regions which can give matching homology with BamHI site sequence (with at least 5 uninterrupted bp) as in Table (16). Practically, three unidirectional-deletion derivatives were obtained in which the deletion started from the BamHI site and continued through in the clockwise direction. The first deletion was about 1290 bp long (Garaev et al., 1982), the second was 1327 bp long (D2) while the third was 1656 bp long (D3). The question arises as to why the other six available homology sequences do not give deletions. This may be due to the need for a long homology sequence in a region adjacent to the first match homology (probably on the other side of the joining endpoints) as found in D2 and D3. However, longer homology sequence (6 and 7 bp) were detected in the same region (Table 16). Albertini et al., (1982) reported that the frequency of deletion formation correlates with the extent of homology between the short repeated sequences, although other factors may be involved. Conley et al., (1986b) showed, by sequential (± 1 bp) changes in the homologous register surrounding the deletion endpoints

Table 16: The probable deletants (unidirectional) which may be produced from pBR322 linearized with BamHI after transformation to E.coli (at least 5 uninterrupted pb were taken in the junction area)^{a,b}.

No.	Homology sequence	Length	No of fit bp		%
			Uninterptd.	Total	Homology
<u>ANTICLOCKWISE</u>					
1	5'-CCGTCCTGTGGATCCT * * * * * 3'-GCGAGTAGCAGTAGGA	102-366 (16.bp)	5	9	56.3
2	5'-CCGTCCTGTGGATCCT * * * * * 3'-GCAGTGGGACCTACGA	124-366 (16 bp)	5	9	56.3
3	5'-CGTCCTGTGGATCCTC ***** * * * 3'-GCAGGTAAGGCTGTCG	190-367 (16 bp)	5	8	50
<u>CLOCKWISE</u>					
4	5'-GCAGGACACCTA ** * * * * * 3'-CGGCCTGTGCGT	367-829 (12 bp)	5	9	75
5	5'-CCTGTGGATCCTCTAC ***** * * * 3'-TCCGACCTACCGGAAG	370-982 (16 bp)	5	8	50
6	5'-GTCCTGTGGATCCTC * * * * * 3'-CTTGACACTTACGCG	368-1346 (15 bp)	5	10	66.7
7	5'-CGTCCTGTGGATCC ***** * * * 3'-GCAGGCGGTAGAGG	367-1391 (14 bp)	5	8	57.1
8	5'-TCCTGTGGAT ***** * * 3'-AGGACAGCAA	369-1469 (10 bp)	6	8	80
9	5'-TCCTGTGGATCCTC * * * * * 3'-ACAAGGCCTAGACG	369-1661 (14 bp)	5	8	57.1 ^c

Table 16: Continued.

No.	Homology sequence	Length	No of fit bp		% Homology
			Uninterptd.	Total	
10	5'-CCGTCCTGTGGATC * ***** 3'-GATGGGACACCTTG	366-1693 (14 bp)	8	10	71.4 ^d
11	5'-TCCTGTGGATCCTCT ** * * * 3'-AGCACTCGTAGGAGA	369-1841 (15 bp)	7	12	80
12	5'-CCTGTGGATCCTCT ***** 3'-AGACACTTAGCGAA	370-2026 (14 bp)	5	9	64.3 ^d
13	5'-GTCCTGTGGATCCTCT * * * 3'-CTGCCACTTTTGGAGA	368-2091 (16 bp)	5	8	50
14	5'-CCGTCCTGTGGATCC ***** 3'-GGCAGTCCCGCGCAG	366-2171 (15 bp)	5	8	53

a- The matching homology sequences were taken from the EcoRI site to near the replication origin (1 - 2500 sites).

b- Computer analysis was done by Mr. Worm (MPI, Mülheim/Ruhr)

c- Deletant obtained by Garaev et al., (1982).

d- Deletants obtained in the present study (unidirectional).

observed, the recombination mechanism was sensitive to (and had a strong preference for) juxtaposition of uninterrupted short homologies. Although they showed that strong homology sequences were needed in both sides of the endpoints for the bidirectional deletion, the results obtained in this study are nearly inverted, since the strong homology on both sides was found in the case of unidirectional deletions.

The nondetected deletants which can be produced from the homology sequences given in Table (16), may be due to the action of exonucleases and or endonucleases. The enzymes will act on a nucleotide region which may contain one or more of the

homology sequences and change the probable deleted endpoints to another homology region. Another possibility is that the other deletants may be produced already in the transformants of our samples but we have chosen only three random deletants (see Figure 7b).

From Table (16) it is noticed that the number of regions having a long homology sequence is lower than those of short homology sequence. This in turn leads us to believe that the probability of producing deletants with endpoints at the long homology sequences is higher than that at short homology sequences. When a shorter length homology sequence (<5 bp) is taken in consideration for the recombination processes, the probability of deletants produced would be greatly increased. Weber and Weissmann (1982) observed that the recombination might require regions of a higher homology. After transformation of E.coli with linear pBR322/SalI and pBR322/PvuII, about 60 and 25 deletants, respectively, have been distinguished (uni and bidirectional) by Conley et al., (1986b).

From the data obtained, it appears that the recombination processes occurring in E.coli for the repairing of the linear plasmid and in turn the formation of deletants, needs a suitable homology sequence (5-8 bp). At the same time all the recombinational processes and subsequent re-cyclization of linear plasmid may be organized by the activities of particular enzymes in the cell. The competitive processes which balance and control the activity of the cell enzymes during the intramolecular recombination of the linearized pBR322 plasmid molecule is important. Albertini et al., (1982) have suggested that the slipped mispairing hypothesis data were consistent with the existence of enzymes that recognize short homologies and catalyse recombination events resulting in deletions. Exonucleases are needed for the removal of the deleted nucleotide sequence and polymerase and ligase are needed for the repair synthesis and cyclization of DNA. The exonuclease III plays a role in re-cyclizing linear plasmid molecules and in the formation of monomeric (Type I) deletion (Conley and

Saunders, 1984 and Conley et al., 1986b). These processes demand exonuclease processing as an essential first step and the alternative deletion end-points could reflect encounters involving the next available homology on recircularizing molecules as exposed by the activities of exonucleases. In addition, the possible cooperative binding of single-strand binding protein (ssbp) to the DNA and endonuclease during recombination processes (Bien, 1986 and Conley et al., 1986b), might also play an active role in the localization of the deletion in plasmid. These factors have been discussed by the latter authors in a proposed mechanism for the formation of the unidirectional deletions (as illustrated in Figure 19b).

A deletion obtained after transformation of E.coli by BamHI linearized plasmid pBR322 was also obtained by Garaev et al., (1982) and Bien et al., (1988) in Amp^r-Tet^s clones. These studies showed that the formation of deleted plasmids does not greatly depend on the system of general recombination of the host bacteria and is not controlled by the recA system. The sequence analysis of Jones et al., (1982) revealed that each deletion event occurred between short homologous or near homologous regions despite the recA nature of the background in which they were formed. However, some studies showed additional details concerning the role of recA in the recircularization processes of the linear DNA. Albertini et al., (1982) demonstrated that the distribution of deletion endpoints derived from recA⁺ and recA⁻ strains were strikingly similar, even though the frequency of deletions in the recA⁻ background was lower. Conley et al., (1986b) showed that the perfect recircularization of linear plasmid was largely recA dependent but the aberrant re-cyclization was depending on the nature of the DNA termini. Other studies showed that the recA protein has an important role in the intramolecular recombination processes of the linear DNA (Cassuto et al., 1980 and Howard-Flanders et al., 1984). Short direct repeats were involved in the formation of deletions and duplications in recA⁺ hosts but for the most part, recA independant recombination had been confined to site-specific events (Warner and Clark, 1980) or to recombination

events associated with transposable elements (Ross et al., 1979). West and Howard-Flanders (1984) illustrated that the recA protein can promote pairing interactions between homologous DNA molecules at a region where both are doublex.

The three unidirectional deletants produced from linear pBR322/BamHI (D2, D3 and that of Garaev et al., 1982) were directed clockwise while those produced from linear pBR322/SalI (by Conley et al., 1986b) anticlockwise. One of the factors which might play a role in the direction of the deletion in deletants produced from linear plasmid is the direction of the cohesive terminus responsible for matching formation. The terminus essential for homology formation and ligation, was found in the anticlockwise direction but the other terminus was not (Fig. 20). Although the termini produced by BamHI are similar to that obtained by SalI (both are cohesive with 4 base-extension) the trend is completely opposite. In the case of SalI, the terminus essential for the homology formation and ligation processes was located at the other direction (clockwise). In this respect, the direction of deletion produced from linear pBR322/BamHI would be inverted to that produced from linear pBR322/SalI as presented in Figure (20).

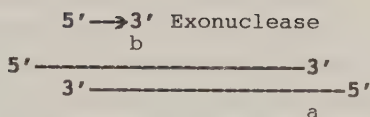
As we show in Figure (19a), the longer strand of the cohesive terminus of the BamHI which is used for recombination invades the other side of the DNA and interacts with the double strands at a position containing a suitable sequence for homologous formation (ternary structure). As mentioned in the literature, this structure is fixed and stabilized by the recA protein. By the action of both exo- and endonucleases the nucleotide sequences (which must be deleted) are removed. The completion of the recombinational steps, will lead to the ligation of the single strand-terminus with the corresponding strand terminus of the other arm of DNA (by ligase). The gap which produced in the daughter strand was then filled (by polymerase) followed by nick sealing (by ligase) to form a new

double stranded plasmid DNA (deletant). However, the trend direction might be opposite in the case of linear pBR322/SalI, since deletion in the two unidirectional deletants, which were sequenced by Conley et al., (1986b), was found to be at the anticlockwise direction.

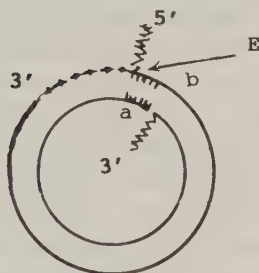
It is not known why one specific terminus is essential for the matching homology formation but not the other. This terminus may be protected from the exonucleases attack by some modification or by coating with specific cell proteins (or enzymes). Conley et al., (1986b) showed that for the perfect recircularization, the end termini of the linear plasmid should be protected from the action of nucleases before repairing. The process might require an enzymatic activity capable of enveloping two exposed anti-parallel single DNA strands (on either end) and promoting a relatively rapid translocation between them. Seroaka and Wackernagel (1977) suggested that the ends of the linear plasmid could be protected from nucleases (even in recBC cells), and the unprotected linear DNA was more frequently degraded than repaired.

Our computer analysis was used for calculating the number of homology sequences (coincide with the corresponding terminus) before (P) and after (F) the restricted site of both BamHI and SalI. The homology sequence was taken for at least 5 uninterrupted bp in the area extended from the EcoRI site to 2500 position. The (P) and (F) values were higher (4 and 21) for SalI terminus than that for the BamHI terminus (3 and 11). However, the P/F ratio was not greatly different (1.9 and 2.7 for SalI and BamHI respectively). The high number of homology sequences corresponds to SalI termini may explain the reason of the relatively short deletion lengths in the two deletants produced after transformation of linear pBR322/SalI as found by Conley et al., (1986b). This may also explain the high number of deletants (about 60) which were electrophoretically distinguished by Conley et al., (1986a) from pBR322/SalI.

BamHI 375
 5'- GGATCCTCTA...
 3'- CCTAGGAGAT...

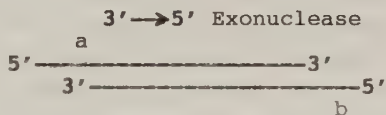


375
 5'- G GATCCTCTA...
 3'- CCTAG GAGAT...
 (AK) (X)

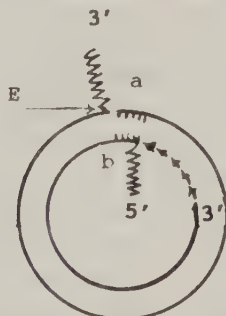


5'- - - - -
 3'- - - - -
 (X)

SalI 651
 ...GAGCGTCGAC -3'
 ...CTCGCAGCTG -5'



651
 ...GAGC GTCGAC -3'
 ...CTCGCAGCT G -5'
 (X) (K)



... - - - - - -3'
 ... - - - - - -5'
 (X)

(as Conley et al., 1986b)

(K) = Clockwise terminus
 (AK) = Anticlockwise terminus
 (X) = End not used in matching
 E = Endonuclease
 a, b = Homology sequence

Figure 20: Simple representation for the possible circularization of the linear pBR322/BamHI and pBR322/SalI plasmids to produce the unidirectional deletions.

The bidirectional deletant D1 was shorter (582 bp) than the previous unidirectional deletants found. Most of the deletion ($\approx 97\%$) was located in the clockwise direction. The matching homology sequence found in this deletant was different than the BamHI termini sequence. Analysis of the number of sequences needed for homology in this case is complicated. The unknown and unlimited sequence which may be responsible for homology formation greatly increases the difficulties of computer analysis for the calculation and characterization of the bidirectional-deletants which may be produced in our samples. The complete disappearance of the BamHI termini found in this deletant may be due to the action of exonuclease which may act on both arms of the DNA strands at the same time. This would lead to the bidirectional removal of the nucleotide sequences (deletion) and would be ended by the action of enzymes required for the recombinational processes when a suitable homology sequence is available. Conley *et al.*, (1986b) showed that linear recombinant plasmid DNA molecules can become re-cyclized *in vivo* by enzymatic removal of protruding termini (produced from the action of PvuI) followed by blunt-ended ligation and only 2 bp were deleted. However, longer bidirectional deletions (650-1800 bp) were obtained after transformation of linear pBR322/SalI, pBR322/PvuII or pBR322/NruI plasmid DNA.

We have noticed that the **G+C** content in the sequences which were responsible for the matching homology formation ($>60\%$) was somewhat higher than that in the ccc-plasmid (54%). This was also found for sequences around the junction points of the detected deletants including the uninterrupted and interrupted homology sequences. It is known that the higher **G+C** content of a DNA, the more stable is it (with a consequent higher melting temperature) since in the **G-C** bp three hydrogen bonds are involved as opposed to two in **A-T** bp. The high **G+C** contents found in the deletants studied, may therefore, play a role in the stabilization of the matching between DNA arms during the recombination processes of the linear plasmid. A high percentage of **G+C** bp (in the area containing the homology

sequence) was also noticed in the deletions of Garaev et al., (1982) and Conley et al., (1986b).

A second point which may be of importance is the occurrence of incomplete *Chi* sites at the areas around the junction points in the deletants studied. In the unidirectional deletants D₂ and D₃, this site sequence (5'-CTGTGG-3' compared to the complete *Chi* sequence of 5'-GCTGGTGG-3') was located just inside the matching homology sequence (370-376). In the bidirectional deletant (D₁), however, the incomplete *Chi* site sequence (5'-GCTGGGCT-3') was located at the other side (arm) from the junction points (946-956) and not included in the homology sequence. This latter findings was also observed by Conley et al., (1986b) in a bidirectional deletant produced from linear pBR322/SalI plasmid transformation into E.coli. The authors reported that this might be fortuitous since such sequences were not encountered in the formation of other deletions. However, Conley and Saunders (1984) proved the involvement of the recB,C product in re-cyclization of plasmid DNA molecules and this enzyme was known to interact with the *Chi* sites. The G+C content and *Chi* sequence observations in addition to the type, size and the specific ends of the homology sequence, may be important factors for the deletants formation and need extensive study to be clarified.

SUMMARY

In this study, the biological consequences of strand breaks in biologically active single-or double stranded viral and plasmid DNA are examined. The damage obtained by irradiation was compared with that produced by restriction endonucleases. The results obtained show that the dose responsible for ssDNA inactivation was about 6-times lower than that for dsDNA. The damaging effect of γ -rays was drastically reduced by the addition of t-butanol as a scavenger for the OH radicals. The ds forms of both ϕ X174 DNA and pBR322 DNA were treated with different restriction endonucleases to form a unique dsb in the molecule. The data obtained show that the majority of linear ϕ X174 DNA molecules were biologically inhibited and only few were recircularized in the cells (0.65-1.7%). With 22-24 Gy of γ -rays, most of the ds ϕ X174 DNA molecules were damaged and only 1-2% activity was obtained. This effect was equal to the action of restricted endonucleases as used for the complete linearization of dsDNA form. The biological activity of the linear dsDNA produced from irradiation was ≈ 8 times higher than that produced by the restriction endonucleases. The yield of transformants produced from the restricted (linear) plasmid DNA ranges from 0.1% to 3.3%. The survival of linear DNA molecules in E.coli cells is the result of a balance between degradation and repair, but repair is rare and degradation is the rule. Results also show that not only is the type of restricted ends of linear plasmid important in determining the repairing efficiency, but so is the base extension and the base sequence of the terminus.

When linear pBR322 DNA molecules were transformed to E.coli, small size-clones (miniclones) were seen to accompany the normal clones on TBV-Ampicillin plates. The miniclones have different sensitivities to the ampicillin and tetracycline, since three types were detected. The first group was resistant to both antibiotics ($\text{Amp}^{\text{r}}\text{-Tet}^{\text{r}}$), the second was sensitive to one antibiotic only ($\text{Amp}^{\text{r}}\text{-Tet}^{\text{s}}$ or $\text{Amp}^{\text{s}}\text{-Tet}^{\text{r}}$) depending on the restricted gene. While the third group, which represents the majority, was sensitive to both antibiotics ($\text{Amp}^{\text{s}}\text{-Tet}^{\text{s}}$). The latter type is probably mainly due to untransformed cells lacking plasmid (satellites) present due to the large initial amount of cells required for transformation. These satellites grow after

β -lactamase release from the central clones. Data showed that the growth-efficiency of miniclones (which contain plasmid) on the antibiotic media was drastically reduced with time. On the other hand, the mutation frequency was increased by long incubation being higher (10-14 fold) than that of corresponding normal clones. A suggested model for plasmid loss was given (50% loss per division). Analysis of the plasmid DNA showed that the transformation of linear plasmid DNA enhanced the formation of plasmid complexes (dimers, trimers or multimers).

A majority ($\geq 95\%$) of the normal transformants obtained contained perfectly recircularized plasmid DNA molecules while a minority contained plasmid with deleted sequences and showed antibiotic sensitivity. Mutation frequency in the transformants ranged from 0.3-12% according to the type of linear plasmid used for transformation. The antibiotic sensitive mutations showed different deletion lengths in their plasmid DNA (100-2000 bp depending on the type of the linear plasmid used). The sequencing of three Tet^S-mutants produced from pBR322/BamHI showed that one deletant had a bidirectional deletion while the others had unidirectional deletions. In the first deletant, 582 bp of DNA were lost while 1327 and 1656 bp were lost in the two unidirectional deletants. The deletion end-points in the two unidirectional deletant plasmids occurred at positions 375 and 1707 bp & 375 and 2036 bp from the EcoRI site respectively. While the end-points in the bidirectional deletant occurred at positions 359 and 942 bp from the EcoRI site. The recyclization point in the deletants was characterized by recombination between directly repeated-homologous sequences of at least five uninterrupted bp on one or both arms of the linearized pBR322. Computer analysis for the homology sequences (coinciding with the sequence-termini) showed some discrimination for the possibility of different deletant productions.

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ZUSAMMENFASSUNG

In der vorliegenden Arbeit werden die biologischen Konsequenzen von Strangbrüchen in biologisch aktiver einzel- und doppelsträngiger viraler DNA und Plasmid-DNA untersucht. Die durch γ -Strahlung induzierten Schäden werden mit den durch Restriktionsendonucleasen prodozierten verglichen. Die Ergebnisse zeigen, daß die zur Inaktivierung von ssDNA benötigte Dosis ca. 6X niedriger ist als für dsDNA. In Gegenwart des OH-Radikalfängers t-Butanol wird die Schädigung durch γ -Strahlung stark reduziert.

Die doppelsträngigen Formen von ϕ X174 und pBR322 DNA wurden mit verschiedenen Restriktionsendonucleasen geschnitten, so daß 1 dsb pro Molekül resultiert. Es zeigt sich für die linearisierten ϕ X174 Moleküle, daß nur ein geringer Teil in den Zellen recircularisiert wird (0.65-1.7%). Bei einer Dosis von 22-24 Gy γ -Strahlung beträgt die biologische Aktivität 1-2%. Die Transformationsrate von enzymatisch linearisierter Plasmid-DNA liegt im Bereich von 0.1% bis 3.3%. Das Überleben von linearisierter DNA in E. coli ist das Resultat zweier Prozesse; Degradation und Reparatur. Die Degradation überwiegt bei diesem Prozeß. Die Ergebnisse zeigen ebenfalls, daß nicht nur die Art der Enden und die Position der Schnittstelle für die Reparatureffizienz eine Rolle spielen sondern auch die Basenüberlappung und die Sequenz der Enden.

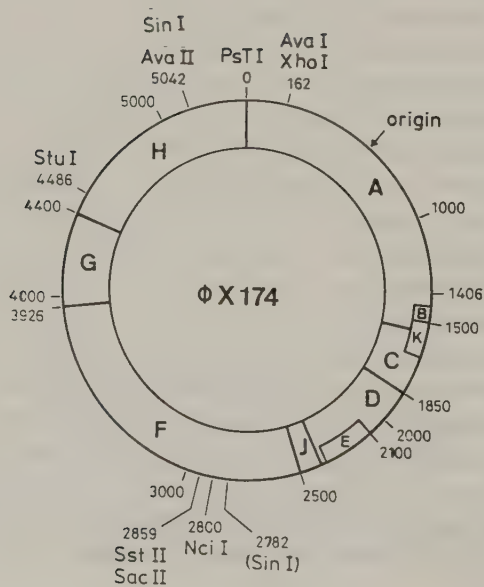
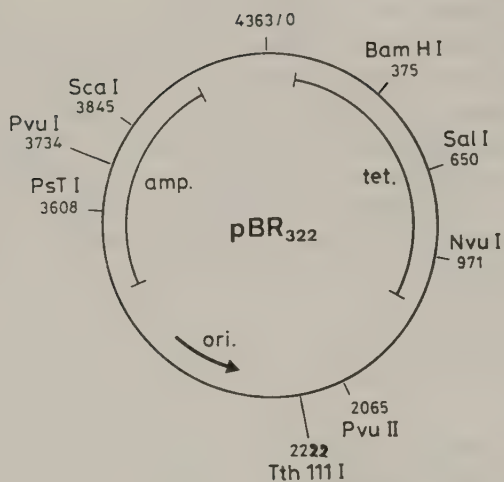
Bei der Transformation von linearisierten pBR322 Molekülen in E. coli traten regelmäßig kleinere Klone (Miniklone) im Vergleich zu den normal-großen auf. Diese Miniklone zeigten sich nach der für Transformationen üblichen Inkubationszeit von 16-20 Std. auf TBY-Ampicillin Platten in der Nachbarschaft der normal-großen Klone. Die Miniklone zeigen unterschiedliche Sensitivitäten gegen die Antibiotika Ampicillin und Tetracyclin. Man kann drei Typen unterscheiden:

- 1- Resistent gegen Ampicillin and Tetracyclin.
- 2- Resistent nur gegen eines der beiden Antibiotika.
- 3- Sensitiv gegen beide Antibiotika.

Die Mehrzahl der kleine Klone gehören zu der dritten Gruppe. Es handelt sich bei diesen sehr wahrscheinlich um nicht transformierte Zellen, die nach Ausscheidung von β -Laktamase durch die transformierten Zellen zu einem langsamen Wachstum fähig sind. Die Wachstumseffizienz von Miniklonen mit Plasmid auf Antibiotikahaltigem Medium reduziert sich drastisch mit der Zeit. Dagegen nimmt die Mutationsrate mit steigender Inkubationszeit zu. Sie ist bei diesen Zellen 10-14X höher als bei den normalgroße Klonen. Ein Modell für den Plasmid-Verlust aus den Zellen wird diskutiert (50% Verlust pro Generation). Die Gelanalyse der Plasmid DNA zeigt, daß bei der Transformation mit linearisierten Plasmiden die Komplexbildung (Dimere, Trimere oder Multimere) zunimmt.

Der Hauptanteil (95%) der normalen Transformanden enthält perfekt recirkularisierte Plasmide. Nur wenige zeigen Deletionen. Die Mutationsrate der Transformanden liegt im Bereich von 0.3-12% abhängig von dem Typus der für die Transformation benutzten linearisierten Molekülen. Die Deletionen betrugen zwischen 100-2000 bp. Die Sequenzierung dreier Tet^S-Mutanten (pBR322/BamHI) zeigte, daß die Deletion einer Mutante in beiden Richtungen verläuft, während die beiden anderen unidirektionale Deletionen aufweisen. Im ersten Fall ergab sich ein DNA Verlust von 582 Basenpaaren, in den beiden unidirektionalen Deletionsmutanten ergab sich ein Verlust von 1327 und 1656 Basenpaaren. Die Endpunkte der Deletionen in diesen Mutanten zeigten sich an den Positionen 375 und 1707 bp und 375 und 2036 bp von der EcoRI site. Für die Recircularisierung linearisierter Plasmid-Molekülen mit Deletionen wird eine homologe Sequenz benötigt. Der Recyclisationspunkt in den unidirektionalen und bidirektionalen Deletionen erfolgte an Sequenzen, die Kurze homologe Sequenzen (5-8 bp) zur Schnittstelle aufweisen. Computer analysen zeigen weiter, daß noch Deletionen als die in dieser Arbeit charakterisieren zu erwarten wären.

APPENDIX

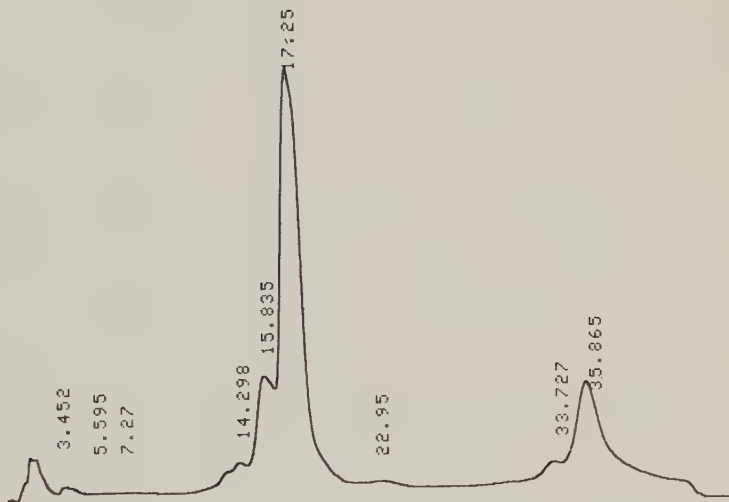


RESTRICTION ENDONUCLEASE CLEAVAGE MAPS OF VIRAL ΦX174 AND PLASMID pBR322 DNA

APPENDIX

WIDTH 5
 DRIFT 0
 T.DBL 0
 ATTN 10
 METHOD\$ 41
 SPL.WT 100
 SLOPE 366.241
 MIN.AREA 10
 STOP.TM 35
 SPEED 3
 FORMAT\$ 0
 IS.WT 1

STOP.TM(3)=45
 START



HIGH PERFORMANCE-LIQUID CHROMATOGRAM OF THE SYNTHESIZED ECORI PRIMER AFTER ITS
 PURIFICATION

CURRICULUM VITAE

NAME: Malak Mahmoud Ismael Zahran
ADDRESS: Masoud Str. 28, Dokki, Cairo Egypt
SEX: Female
BIRTH: April 26, 1952, Cairo Egypt
NATIONALITY: Egyptian

EMPLOYMENT HISTORY:

1959 - 1965 Primary school, Cairo Egypt
1965 - 1967 Preparatory school, Cairo Egypt
1967 - 1970 Secondary school, Cairo Egypt
1970 - 1975 Faculty of Agriculture, Cairo University.
1975 B.Sc. in Biochemistry, Faculty of Agriculture, Cairo University.
10/1975 - 7/1976 Air Hostess, Bavaria Airlines rented to Egypt Air Company, Cairo Egypt.
10/1976 - 5/1977 Training in the Middle Eastern Regional Radioisotope Centre for Arab Countries.
1/1978 - 5/1982 Research Fellow, National Centre for Radiation Research and Technology, Nasr City, Cairo Egypt.
1982 M.Sc. in Biochemistry, Faculty of Agriculture, Cairo University.
6/1982 - 9/1984 Assistant Lecturer in National Centre for Radiation Research and Technology, Nasr City, Cairo Egypt.
10/1984 - 3/1985 German language studying, Goethe Institut , Göttingen, West Germany.
4/1985 - 3/1989 Ph.D. Student in Max-Planck-Institut für Strahlenchemie, Mülheim a.d. Ruhr (registered in Faculty of Biology, Ruhr-Universität Bochum).

